THE

BIOCHEMICAL JOURNAL

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Concentration of Sugars, Ketone Bodies and some Organic Acids in Udder Lymph and Plasma of Cattle

BY G. V. HEYNDRICKX* AND G. J. PEETERS
State Agricultural University, Ghent and Veterinary College, State University, Ghent, Belgium

(*Received* 15 *June* 1959)

Heyndrickx & Peeters (1958*a*, *b*) and Heyndrickx (1959) have described the electrolyte, enzyme, lipid and protein composition of the udder lymph and blood plasma in cattle. Because of the importance of the udder lymph in the synthesis of milk, the present paper extends their findings to include the sugars, ketone bodies and some organic acids.

MATERIALS AND METHODS

Biological material. East Flemish and Friesian cows in good lactation and with normally healthy udders were used. A blood sample was taken from the udder veins and, after slaughter of the animal, the udder lymph was sampled as previously described (Heyndrickx & Peeters, 1958*a*). Blood and lymph were heparinized and then centrifuged. The interval elapsing between taking the blood and lymph samples was about 15 min.

Analytical methods

Sugars. These were estimated by circular and descending chromatography with the solvent mixtures butanol–acetic acid–water (4:1:5, by vol.) and phenol (Partridge, 1948). The samples were first deproteinized and desalted as described by Odin & Werner (1949) and Wallenfels, Bernt & Limberg (1953). Glucose and lactose were determined on the developed chromatograms with *o*-aminodiphenyl (Piper & Bernardin, 1958). Fructose was identified by comparison with a control chromatogram and determined after extraction by the method of Honer & Tuckey (1953). Since the content of fructose is very low in lymph and plasma a sensitive method is essential.

Ketone bodies. Acetone plus acetoacetic acid was determined with 2:4-dinitrophenylhydrazine according to Mayes & Robson (1957). Acetoacetic acid was determined by the method of Walker (1954) and the free acetone was calculated by difference.

Organic acids. Pyruvic acid and α-oxoglutaric acid were estimated by chromatography of the 2:4-dinitrophenylhydrazones (Cavallini & Mondovi, 1957); the solvent systems phenol (Partridge, 1948) and 0·1 N-glycine buffer, pH 8·4 (Virtanen, Miettinen & Kunttu, 1953), were used. Citric acid and lactic acid were determined by the method of Natelson, Pincus & Lugovoy (1948) and Barker & Summerson (1941) respectively. The results obtained were in agreement with the semi-quantitative paper-chromatographic methods of Nordmann, Marty, Tholozan & Nordmann (1957) and Osteux & Laturaze (1956).

Proteins. Protein content was calculated from the refractive index (Heyndrickx, 1959).

The results have been evaluated statistically according to Snedecor (1950). To calculate the significance of the difference between the means for each constituent in lymph and plasma, each of the plasma samples was paired with a corresponding sample of the lymph taken from the same cow, and the paired *t* test was used.

RESULTS

Table 1 shows the mean values and standard deviations for 11 samples of udder lymph and of the corresponding plasma from the udder vein; the degree of significance of the difference between the means of each constituent is also mentioned.

Sugars. The mean concentration of glucose in lymph was significantly lower than that in plasma. The figures for plasma confirm the results of Barros (1942) and Bach & Hibbit (1959). The content of fructose was lower in lymph than in plasma, but lactose is present in higher concentration in lymph.

Ketone bodies. The acetone concentration is higher in lymph than in plasma, although the concentration of acetoacetic acid is practically the same in the two fluids.

Organic acids. Pyruvic acid occurs in lower concentration in lymph than in plasma, whereas the reverse is true for α-oxoglutaric acid, citric acid and lactic acid. The concentrations in the plasma of the second and third acids are in agreement with the values reported by Blosser, Niedermeier & Smith (1951), Van den Berg (1946) and Powell & Shaw (1942).

Proteins. In agreement with our previous work, the protein content was much lower in lymph than in plasma.

DISCUSSION

There is no literature on the composition of udder lymph, but Yoffey & Courtice (1956) write that the readily diffusible non-protein constituents of the plasma are present in the lymph from other sources in concentrations which are approximately the same as in the plasma. Our results, however, show that in udder lymph the concentrations of most of the constituents differ significantly from those in plasma.

* Associé du Fonds National de la Recherche Scientifique.

Table 1. *Mean composition of 11 samples of udder lymph and plasma of the udder veins*

Constituent	Mean concn. (mg./100 ml.)±S.D. Lymph	Plasma	*P* at which concn. in lymph – concn. in plasma is significant
Glucose	48·2±11·0	70·6±12·1	<0·01
Fructose	0·65±0·43	1·16±0·64	0·01
Lactose	12·5±7·6	5·3 ±5·1	<0·01
Acetone	0·46±0·48	0·31±0·31	0·1
Acetoacetic acid	0·19±0·14	0·22±0·12	0·4
Pyruvic acid	0·42±0·07	0·71±0·34	0·02
α-Oxoglutaric acid	0·17±0·07	0·10±0·05	<0·01
Citric acid	4·05±1·67	2·63±1·32	<0·01
Lactic acid	87·6±15·2	7·0 ±3·6	<0·01
Protein (g./100 ml.)	2·86±1·00	8·41±0·66	<0·01

The cow was always 'milked out' a short time before slaughter, as otherwise the lymph, which had been punctured from an udder abnormally tense with milk, was very turbid instead of being clear and contained the constituents of the milk in high concentrations. This shows that considerable reabsorption of milk by the lymphatics takes place under these conditions. Some reabsorption of readily diffusible milk constituents may occur under normal conditions. It is well known that urine and serum from pregnant and lactating women may contain lactose (Archer & Haram, 1948; Odin & Werner, 1949) and the higher concentration of lactose in lymph relative to plasma may similarly be explained. The higher concentration of citric acid and α-oxoglutaric acid in lymph may also be due to reabsorption from the milk, in which the average concentrations of citric acid and α-oxoglutaric acid are respectively 181 (Schwarz & Mumm, 1953) and 0·24 mg./100 ml. (Harper & Huber, 1956). The occurrence of α-oxoglutaric acid and citric acid in the udder lymph in higher concentrations than in the mammary-vein plasma would appear to provide additional evidence of the occurrence of the Krebs cycle in the mammary gland, as first demonstrated by Terner (1951).

Many investigations have shown that the blood glucose is the principal precursor of the lactose of the milk (Dimant, Smith & Lardy, 1953; Kleiber *et al.* 1955). Peeters, Sierens, Tindal & Coussens (1955) have found that during perfusion of the isolated mammary gland of the cow considerable quantities of glucose and fructose disappeared from the blood. It is probable that this may be related to the lower concentrations of glucose and fructose found in lymph relative to the plasma. Shaw (1946) shows no significant difference in pyruvic acid between arterial and venous blood samples from the cow's mammary gland. Peeters, Coussens & Sierens (1954) have found a decrease of the blood pyruvic acid on perfusion of the isolated cow mammary gland in the presence of fluoroacetate. The chemical methods employed by these workers for determination of pyruvic acid are, however, not highly specific. It is possible that pyruvic acid is utilized by the mammary gland and in this case the lower content of this acid in lymph compared with plasma may be explained in the same way as for glucose and fructose. In milk the concentration of pyruvic acid, like that of glucose and of fructose, is very low (0·008 mg./100 ml. according to Harper & Huber, 1956).

It is very striking that the concentration of lactic acid is about 12 times as high in lymph as in plasma. This is not explicable by a reabsorption from the milk since the concentration of lactic acid in milk is about 2 mg./100 ml., of the same order as that in plasma. On the other hand, it is known that mammary tissue produces lactic acid on incubation (Knodt & Petersen, 1945) and it is possible that this diffuses into the lymph.

SUMMARY

1. Sugars, ketone bodies and organic acids were determined in udder lymph and plasma from the veins of the udder of cattle.

2. The concentrations of glucose and fructose are lower and that of lactose is higher in lymph than in plasma.

3. The free acetone content attains higher values in lymph than in plasma, although the concentration of acetoacetic acid is practically the same in the two fluids.

4. The α-oxoglutaric acid, citric acid and especially lactic acid contents attain significantly higher values in lymph than in plasma; the pyruvic acid content of lymph is, however, below that of plasma.

REFERENCES

Archer, H. E. & Haram, B. J. (1948). *Lancet*, i, 558.
Bach, S. J. & Hibbit, K. G. (1959). *Biochem. J.* **72**, 87.
Barker, S. B. & Summerson, W. H. (1941). *J. biol. Chem.* **138**, 535.
Barros, L. A. (1942). *Arch. Soc. Biol. Montevideo*, **10**, 308.
Blosser, T. H., Niedermeier, R. P. & Smith, V. R. (1951). *J. Dairy Sci.* **34**, 75.
Cavallini, D. & Mondovi, B. (1957). *Clin. chim. Acta*, **2**, 312.
Dimant, E., Smith, V. R. & Lardy, H. A. (1953). *J. biol. Chem.* **201**, 85.
Harper, W. J. & Huber, R. M. (1956). *J. Dairy Sci.* **39**, 1609.
Heyndrickx, G. V. (1959). *Quart. J. exp. Physiol.* **44**, 264.
Heyndrickx, G. V. & Peeters, G. (1958*a*). *Quart. J. exp. Physiol.* **43**, 174.
Heyndrickx, G. V. & Peeters, G. (1958*b*). *Enzymologia*, **20**, 161.
Honer, C. J. & Tuckey, S. L. (1953). *J. Dairy Sci.* **36**, 1233.
Kleiber, M., Black, A. L., Brown, M. A., Baxter, C. F., Luick, J. R. & Stadtman, F. H. (1955). *Biochim. biophys. Acta*, **17**, 252.
Knodt, C. B. & Petersen, W. E. (1945). *J. Dairy Sci.* **28**, 415.
Mayes, P. A. & Robson, W. (1957). *Biochem. J.* **67**, 11.
Natelson, S., Pincus, J. & Lugovoy, J. (1948). *J. biol. Chem.* **175**, 745.
Nordmann, R., Marty, A., Tholozan, A. & Nordmann, J. (1957). *C.R. Acad. Sci., Paris*, **245**, 2414.
Odin, L. & Werner, I. (1949). *Acta chem. scand.* **3**, 989.
Osteux, R. & Laturaze, J. (1956). *Clin. chim. Acta*, **1**, 378.
Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
Peeters, G., Coussens, R. & Sierens, G. (1954). *Arch. int. Pharmacodyn.* **98**, 288.
Peeters, G., Sierens, G., Tindal, J. & Coussens, R. (1955). *Arch. int. Pharmacodyn.* **102**, 470.
Piper, C. V. & Bernardin, L. J. (1958). *Tech. Pap. Pulp Pap. Ind., N.Y.*, **41**, 16.
Powell, R. C. & Shaw, J. C. (1942). *J. biol. Chem.* **146**, 207.
Schwarz, G. & Mumm, H. (1953). *Kiel. Milchw. ForschBer.* **5**, 511.
Shaw, J. C. (1946). *J. Dairy Sci.* **29**, 183.
Snedecor, G. W. (1950). *Statistical Methods*. Ames, Iowa: State College Press.
Terner, C. (1951). *Biochem. J.* **50**, 145.
Van den Berg, W. (1946). *Tijdschr. Diergeneesk.* **71**, 497.
Virtanen, A. I., Miettinen, J. K. & Kunttu, H. (1953). *Acta chem. scand.* **7**, 38.
Walker, P. G. (1954). *Biochem. J.* **58**, 699.
Wallenfels, K., Bernt, E. & Limberg, G. (1953). *Angew. Chem.* **65**, 581.
Yoffey, J. M. & Courtice, F. C. (1956). *Lymphatics, Lymph and Lymphoid Tissue*. London: Edward Arnold Ltd.

Biochem. J. (1960) **75**, 3

The Synthesis of Methionine from Homocysteine by Enzymic Extracts of *Escherichia coli*

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In earlier work from this Laboratory it was shown that washed suspensions of various strains of *Escherichia coli* synthesize methionine from homocysteine (Gibson, 1952; Gibson & Woods, 1952, 1960). With strains unable to synthesize serine evidence was obtained that this amino acid was the probable ultimate source of the C_1 fragment for the methylation. With strains requiring *p*-aminobenzoic acid for growth it was shown that this factor was essential for methionine formation and there was strong, though indirect, evidence that it must first be converted into some form of folic acid. The purpose of the present work was to explore the probable function of folic acid under the more closely defined conditions possible with cell-free enzymic extracts and to determine other cofactor requirements.

In exploratory experiments published briefly by Cross & Woods (1954) it was found that enzymic extracts made by grinding the organisms with alumina synthesized methionine from homocysteine and serine in a reaction mixture containing also hexose diphosphate, pyridoxal phosphate, adenosine triphosphate and Mg^{2+} ions; preparations made by ultrasonic disintegration or by shaking with minute glass beads were less active. A heated extract of *E. coli* increased methionine synthesis by about half. It was later found (by Dr M. J. Cross and the present authors, unpublished work) that extracts of acetone-dried organisms were a more potent and reliable source of the enzyme systems concerned. The problem was therefore investigated in detail with this material. Part of this work has been the subject of a preliminary communication (Szulmajster & Woods, 1955).

METHODS AND MATERIALS

Growth of organisms

Escherichia coli PA 15 was used throughout the work. It is an auxotrophic strain requiring either serine or glycine for growth and was originally obtained from Dr Barbara Wright. Stock cultures were maintained on slopes of tryptic meat–agar, subcultured monthly, incubated for 16 hr. at 37° and stored at 4°.

For large-scale growth the basal medium contained (g./l.): K_2HPO_4, 9·6; KH_2PO_4, 4·4; $(NH_4)_2SO_4$, 1·0; NH_4Cl, 2·5; $MgSO_4,7H_2O$, 0·1; glucose, 2·0; lactic acid, 10; glycine 0·75. After addition of Fe^{2+} ions (final concn. 4 μM), from a solution of $FeSO_4,(NH_4)_2SO_4,6H_2O$ in twice its molar concentration of sodium citrate, the pH was brought to pH 7·3 with 10 N-NaOH. Basal medium (500 ml.) and powdered agar (15 g.) were autoclaved in enamelled trays (38 cm. × 25 cm. × 3 cm.) at 108° for 5 min. and then brought to 126° for 20 min. Each tray was inoculated with sufficient organisms (about 1 mg. dry wt.) to ensure confluent growth; the inoculum was derived from Roux bottles containing the same agar medium and incubated for 18 hr. The trays, usually in batches of 200–400, were incubated at 37° for 40 hr.

The harvested organisms (about 0·8–1·0 g. dry wt./tray) were made into a smooth suspension with a little water in a ball mill. After filtering through glass wool to remove particles of agar, the suspensions were stored at 0° in full screw-capped bottles.

Smaller batches of organisms which were occasionally required were grown in liquid culture in the above-described basal medium (500 ml.) with vigorous shaking for 16 hr. at 37°; the inoculum was 5 ml. of a culture (24 hr., 37°) on the same medium. The organisms were washed twice on the centrifuge with one-tenth of culture volume of 0·067 M-phosphate buffer, pH 7·2.

Preparation of enzymic extract

Organisms harvested from 200 trays (about 170–200 g. dry wt.) were washed twice on a Sharples centrifuge with 5 l. quantities of 0·067 M-phosphate buffer, pH 7·1. The final sediment was suspended with vigorous stirring in acetone (5 l.) at −20° and the suspension was filtered through a Büchner funnel. The operation was repeated with a further 5 l. of acetone and, after washing four times with acetone (400 ml.), the cake of organisms was placed in a desiccator over P_2O_5 and the desiccator was evacuated for several hours to remove residual acetone. The organisms were left in the closed desiccator overnight and finally stored at −15° in sealed containers. The acetone-dried powder has been kept for 8–10 months without loss of activity.

Samples of the acetone-dried organisms were intimately mixed for 20 min. (in a Potter–Elvehjem homogenizer) with ten times the powder volume of either water or 0·067 M-phosphate buffer, pH 7·1, at 25°. The supernatant after centrifuging for 20 min. at 16 000 *g* was stored at 0° and will be referred to as crude enzymic extract.

For most experiments the crude extract was first dialysed for 18–24 hr. against running tap water; there was some loss of activity (up to 20%) even when necessary cofactors were added.

A unit of enzyme is defined as that quantity catalysing the formation of 1 μm-mole of L-methionine in 3 hr. Specific activity is expressed as μm-moles of L-methionine formed/mg. of protein/hr.

Extract of heated organisms

Washed or acetone-dried organisms (each 1 g.) were suspended in 6 ml. of water, heated for 3 min. at 100° and centrifuged for 20 min. at 16 000 *g*. The supernatant will be referred to as extract of heated *E. coli*; it contained about 20 mg. dry wt./ml. In the final stages of the work the heated extract was diluted with an equal volume of acetone, the precipitate removed and the supernatant lyophilized. All three methods of preparation gave material with similar properties.

Reaction mixture for the synthesis of methionine

The complete reaction mixture in which synthesis of methionine was tested contained (in 2 ml. final volume in 125 mm. × 16 mm. tubes): enzyme preparation (4–5 mg. of protein); DL-serine (10 μmoles); DL-homocysteine (20 μmoles); diphosphopyridine nucleotide (DPN; 0·5 μmole); adenosine triphosphate (ATP; 7 μmoles); $MgCl_2$ (5 μmoles); extract of heated *E. coli* (EHC; 0·2 ml.); phosphate buffer, 0·02 M, pH 7·2. The tubes were normally incubated for 3 hr. at 37°. Any deviations from these standard conditions are noted in the Results section.

The reaction was stopped by placing the tubes in boiling water for 3 min.; the coagulated protein (which retained about 5–10% of the total methionine) was removed by centrifuging and the supernatant was used for assay of methionine.

A control in which the complete reaction mixture was heated to 100° for 5 min. at zero time was included in each experiment.

Assay of methionine

With *Leuconostoc mesenteroides* P 60 the procedure of Gibson & Woods (1960) was followed exactly.

Assays with *Streptococcus faecalis* (which were used in the latter part of the work) have the advantage that incubation need be for only 16–17 hr., compared with 44–48 hr. with *L. mesenteroides*. L-Methionine must, however, be used as standard since the organism contains a racemase which converts the D- into the L-isomer (Shockman & Toennies, 1954). Results with the two organisms showed satisfactory agreement.

The strain used was American Type Culture Collection no. 9790. The basal medium of Toennies & Shockman (1953) was used, but the glucose concentration was raised to 4% (w/v) and one-tenth of the culture volume of peroxide-treated peptone was added to increase the growth rate. For preparation of the latter, Bactopeptone (Difco) (50 g.) was added to 460 ml. of water, 42 ml. of 10 N-HCl and 5·1 ml. of H_2O_2 (100 vol.). After standing for 24 hr. at room temperature the solution was filtered, heated for 30 min. at 100°, adjusted to pH 6·7 with 10 N-NaOH, diluted to 1 l. and stored at 2°.

The assay was carried out in final culture volumes of 2 ml.; sterilization was by autoclaving for 5 min. at 108°. The inoculum was 0·1 ml. of a 6 hr. culture on the same medium containing just the optimum concentration of methionine. After incubation at 37° (in a water bath) for 16–17 hr., growth was assessed and interpreted as in the *L. mesenteroides* assay (Gibson & Woods, 1960).

Other estimations

Glycine. This was determined by the method of Alexander, Landwehr & Seligman (1945); the accuracy in the present work was about ±15–20%.

Protein. For intact organisms the biuret method of Stickland (1951) was used. Protein in enzymic extracts was determined turbidimetrically after precipitation with trichloroacetic acid (Stadtman, Novelli & Lipmann, 1951); crystalline serum albumin was used as standard.

Chromatography

Ascending and descending chromatograms were run on Whatman no. 1 or no. 4 filter paper. Developing agents used were: (*a*) propanol–pyridine–water (1:1:1, by vol.); (*b*) butanol–propionic acid–water (prepared by mixing equal volumes of 84 ml. of water in 1246 ml. of butanol and 620 ml. of redistilled propionic acid in 730 ml. of water); (*c*) acetone–triethylamine–water (80:5:15, by vol.). The last-named solvent was used for the separation of glycine from serine (Wright & Stadtman, 1956). The detecting agent for amino acids was ninhydrin.

Chemicals

Folic acid derivatives. Tetrahydropteroylglutamic acid was prepared by catalytic hydrogenation of pteroylglutamic acid (O'Dell, Vandenbelt, Bloom & Pfiffner, 1947). A specimen of *N*-formylpteroyltriglutamic acid isolated from *Clostridium cylindrosporum* by Dr Barbara Wright was kindly provided by her. Teropterin (pteroyl-γ-glutamyl-γ-glutamylglutamic acid) was a gift from Dr T. H. Jukes. Other folic acid compounds were as described by Lascelles, Cross & Woods (1954).

Other chemicals. Pyridoxal phosphate was a gift from Dr K. Folkers and thiomethyladenosine from Dr F. Schlenk. ATP (potassium salt) was either prepared from rabbit skeletal muscle (LePage, 1949) or obtained commercially. Other chemicals were commercial products, recrystallized when necessary.

Potassium phosphate buffers were used throughout.

Isotope experiments

Estimates of radioactivity were made in an end-window Geiger–Müller counter with dry samples in flat-bottomed aluminium planchets. Reported values (net counts) have been corrected for self-absorption by using a self-absorption curve for $BaCO_3$. Sufficient counts were made to give a standard error of ±5%.

Reaction products were brought to pH 2·0 and separated into anionic and cationic fractions by chromatography on ion-exchange resin (Dowex-50, H^+ form). Labelled methionine was recovered from the cationic fraction by addition of 10 μmoles of unlabelled L-methionine as carrier and isolation by paper chromatography. The methionine spot was eluted and counted. (The radioactive precursor added was serine; R_F values of methionine and serine are 0·58 and 0·36 respectively in propanol–pyridine–water.)

The methyl group was removed from methionine by the method of Vieböck & Brecher (1930) in a micro-Zeisel apparatus; the methyl iodide was trapped in cold acetone containing *iso*thiourea. The methyl *iso*thiouronium salt was detected on paper chromatograms by the method of Večeřa & Gasparič (1954) and, after elution, the radioactivity of the chromatographic spot was determined.

RESULTS

General properties of the crude enzyme extract

The activity of extracts of acetone-dried organisms made with phosphate buffer (0·067 M), pH 7·1, was twice that of those made with either water or sodium bicarbonate (0·067 M). With the crude enzymic extract a net synthesis of methionine occurs in phosphate buffer with homocysteine and serine only (Table 1); this was increased by 50–100% by addition of an extract of heated *E. coli* (EHC). The following had no effect either in the presence or in the absence of EHC: ATP, DPN, pyridoxal phosphate, pteroylglutamic acid and its N^5-formyltetrahydro derivative (leucovorin).

The crude extracts contained about 15–17 mg. of protein/ml. and had a specific activity (no added EHC) of 20–30 μm-moles of L-methionine/mg. of protein/hr. The cell debris remaining after extraction had no activity. No active material was sedimented from the extracts by centrifuging for 1 hr. at 80 000 *g*.

Table 1. *Comparison of methionine synthesis by whole organisms and enzymic extracts*

Whole organisms were incubated for 4 hr. at 37° in 2 ml. of a reaction mixture which contained (in addition to substances indicated): bacterial suspension (0·1 ml., equivalent to 0·4 mg. of protein); glucose (0·05 M); *p*-aminobenzoic acid (10 μM); pyridoxal phosphate (2·5 μM); cobalamin (10 μmg./ml.) and phosphate buffer (0·01 M, pH 7·3). Crude enzymic extract was incubated for 3 hr. at 37° in 2 ml. of a reaction mixture which contained (in addition to substances indicated): enzymic extract (0·4 ml., equivalent to 5 mg. of protein) and phosphate buffer (5 mM, pH 7·3). EHC, Extract of heated *E. coli* (see Methods section). +, Present; −, absent.

Source of enzyme	Additions to reaction mixture: Homocysteine (0·1 M)	Serine (0·1 M)	EHC (0·2 ml.)	L-Methionine formed (μm-moles/mg. of protein/hr.)
Intact organisms	−	−	−	25
	−	+	−	40
	+	+	−	438
Crude enzymic extract	−	−	−	0
	−	+	−	7
	+	+	−	23
	+	+	+	37

Relative activity compared with whole organisms. The specific activity of the crude extract, even with EHC added, was only about 7 % of that of the original organisms (Table 1). This may result from destruction of cellular organization or from the presence of some enzyme or cofactor in limiting quantities only.

Stability. The extract lost little or no activity if kept at 0° for 2 weeks or at −15° for several months. Lyophilization, however, led to a 70 % loss. It is relatively stable to heat, the loss being only 50 % in 30 min. at 45° (pH 7·1).

Other enzymic activities. The crude extract did not destroy methionine; the relative lack of activity compared with whole organisms cannot therefore be due to this. No evidence for homocysteine desulphydrase activity was found by searching for its product (α-aminobutyric acid) on paper chromatograms. The extract contained a weak serine deaminase; however, only 8 % of serine present in the standard reaction mixture for methionine synthesis was converted into pyruvic acid.

Enzyme and substrate concentrations. The rate of methionine formation increased with enzyme concentration up to the equivalent of about 5 mg. of protein/ml. (Fig. 1). The approximate concentrations of serine and homocysteine for half-maximum rate of synthesis of methionine were 1 and 3 mM respectively (Fig. 2); the value for homocysteine is reduced to 1 mM if anaerobic conditions are used, suggesting that homocystine is not metabolized.

pH. The optimum pH under the present conditions is not sharp; over 90 % of the optimum methionine synthesis was obtained in the range pH 7·0–8·5 (Fig. 3).

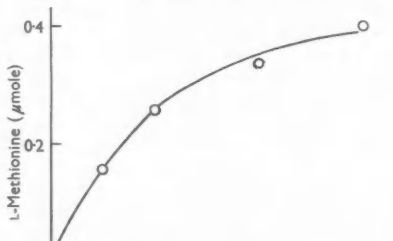

Fig. 1. Effect of concentration of enzymic extract. The reaction mixture (2 ml.) contained DL-serine (20 μmoles) and DL-homocysteine (20 μmoles) in 0·2 M-phosphate buffer, pH 7·2; crude enzymic extract was added as indicated. Incubation was for 3 hr. at 37°.

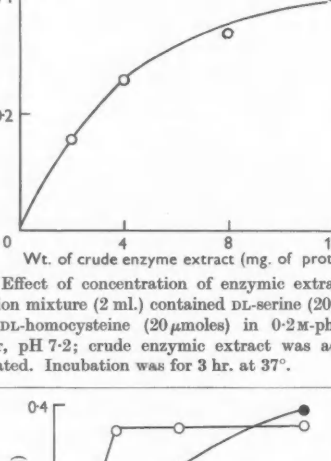

Fig. 2. Effect of concentration of substrates. The reaction mixture (2 ml.) contained 0·3 ml. of crude enzymic extract (equivalent to 4·5 mg. of protein) in 0·02 M-phosphate buffer, pH 7·2, with either DL-serine or DL-homocysteine (both 20 μmoles). The other amino acid (DL-serine, ○; DL-homocysteine, ●) was added as indicated. Incubation was for 3 hr. at 37°.

Dialysed enzyme extract

A requirement for several cofactors besides extract of heated *E. coli* (EHC) could be demonstrated if the crude extracts were dialysed 18–24 hr. against running tap water (Table 2); furthermore the effect of EHC was sharpened.

Inorganic phosphate. Replacement of phosphate buffer in the standard reaction mixture by tris (2-amino-2-hydroxymethylpropane-1:3-diol) buffer of the same pH and molarity led to a 75 % decrease in methionine synthesis (Table 3); the residual activity may be due to phosphate present in EHC. Activity was restored by the addition of phosphate (Table 3); it was shown that this was not due to the Na^+ and K^+ ions added consequentially.

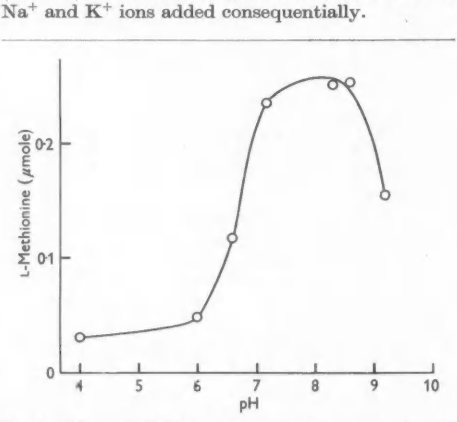

Fig. 3. Effect of pH. The reaction mixture contained crude enzymic extract (equivalent to 5 mg. of protein), DL-serine (20 μmoles) and DL-homocysteine (20 μmoles) in 0·02 M-buffer of the pH indicated. Nature of buffer: succinate (pH 4·0–6·0); phosphate (pH 6·5–7·5); tris (pH 8·0–9·2). Phosphate (40 μmoles, of corresponding pH) was added in each case to satisfy the requirement for this ion.

An attempt was made, with isotope technique, to determine if the phosphate was required for the initial phosphorylation of one of the main substrates (homocysteine and serine) participating in methionine formation; [^{32}P]orthophosphate of high specific activity ($1{\cdot}06 \times 10^5$ counts/min./μmole) was added to the standard reaction mixture. The organic phosphate fraction contained about 40% of the original radioactivity. The cation fraction (which contains the amino acids) was separated on Dowex-50 resin but contained no radioactivity. With the limitations set by possible lability of the compounds it was provisionally concluded that phosphate is not concerned in a phosphorylation of serine or homocysteine; it may be required, among other things, for the regeneration of ATP, since adenosine pyrophosphatase is present in the extracts.

Diphosphopyridine nucleotide. Omission of DPN from the standard reaction mixture decreased methionine synthesis to about 40% (Table 2). Maximum activity was obtained with about 0·3 μmole/ml. When the enzymic extract was first incubated with nicotinamide (0·5 mM) the DPN requirement for subsequent methionine synthesis was decreased by 20–40%.

Adenosine triphosphate and magnesium ion. The joint omission of these substances reduced synthesis of methionine to about 40% in the experiment of Table 2; in another experiment (Table 4), in which dialysis was more exhaustive, a greater reduction was obtained. There was very little extra activity when either substance was added without the other. For maximum activity it was necessary to add them in a definite molar ratio (1·2 ATP : 1 Mg^{2+} ion); a greater or lesser ratio gave markedly less synthesis (Table 4). Adenosine diphosphate did not replace ATP.

A similar relationship between ATP and Mg^{2+} ions has been found by Kuby, Noda & Lardy (1954) for ATP-creatine phosphorylase. It is supposed that a complex of Mg^{2+} ions and ATP rather than free ATP is the metabolite and that competition between the enzyme and ATP for the Mg^{2+} ions may occur.

Pyridoxal phosphate. No effect of this coenzyme was found even after exhaustive dialysis of the crude enzymic extract. Some stimulation (20–40%) was occasionally obtained if both the enzyme and the EHC were first irradiated (sunlight) but the effect was not reproducible. An effect of pyridoxal phosphate had been observed by Cross & Woods (1954) with an enzyme preparation made by grinding with alumina. Absorption of the present dialysed extract with alumina led to a small but reproducible degree of resolution; in two experiments addition of pyridoxal phosphate (20 μM) to the supernatant after such treatment increased methionine synthesis from 0·13 and 0·10 to 0·16 and 0·12 μmole respectively.

Extract of heated Escherichia coli. The effect of EHC observed with crude enzymic extracts (Table 1) was much more pronounced with well-

Table 2. *Requirements for the synthesis of methionine*

Substances were omitted as indicated from the standard reaction mixture (see Methods section). Source of enzyme: 0·4 ml. (4 mg. of protein) of dialysed enzymic extract. Incubation was for 3 hr. at 37°.

Substance omitted	L-Methionine formed (μm-moles)
None	190*
DL-Serine	20
DL-Homocysteine	10
EHC	40
DPN	65
ATP + Mg^{2+} ions	80
Phosphate	40

* With crude undialysed enzymic extract this figure was 205.

Table 3. *Effect of orthophosphate on methionine synthesis*

Standard reaction mixture (containing dialysed enzymic extract equivalent to 4 mg. of protein) was used except that phosphate buffer was omitted and replaced by phosphate or tris buffers of the same pH (7·2) and the molarities indicated. Incubation was for 3 hr. at 37°. +, Present; −, absent.

Phosphate buffer (mM)	Tris buffer (0·02 M)	L-Methionine formed (μm-moles)
20	−	141*
−	+	31
5	+	65
10	+	103
20	+	138

* This figure is reduced to 33 if EHC is omitted.

Table 4. *Effect of adenosine triphosphate and Mg^{2+} ions on methionine synthesis*

Standard reaction mixture (containing dialysed enzymic extract equivalent to 4 mg. of protein) was used except that ATP and Mg^{2+} ions were added at the concentrations indicated. Incubation was for 3 hr. at 37°. Results (relative activity) are expressed as percentages of the highest value.

ATP (mM)	Mg^{2+} ions (mM)	Molar ratio ATP/Mg^{2+} ions	Relative activity
0	0	—	5
3	0	—	16
0	2·5	—	18
3	2·5	1·2	100
3	0·5	6·0	15
3	1	3·0	23
20	2·5	8·0	71
6	10	0·6	54
6	5	1·2	96

dialysed extracts; methionine synthesis was reduced to 25% in its absence even with ATP, Mg^{2+} ions, DPN and PO_4^{3-} ions present (Table 2). The relationship of effect to concentration of EHC was not linear (Fig. 4); possibly inhibitory material is also present.

It appeared likely that at least one component of EHC would be a coenzyme form of folic acid. With whole organisms of another strain of *E. coli* Gibson & Woods (1960) found strong presumptive evidence that *p*-aminobenzoic acid (which is required for methionine synthesis) must first be converted into some form of folic acid. Tetrahydropteroylglutamic acid, albeit at relatively high concentrations, is an effective cofactor for transfers of C_1 units in the serine–glycine reaction and in purine formation (see review by Wright, 1958) and has recently been shown by Nakao & Greenberg (1958) to function in the overall methylation of homocysteine by serine or formaldehyde with sheep-liver homogenates. However, neither tetrahydropteroylglutamic acid nor its N^5-formyl derivative (leucovorin) had any activity in the present system in replacing EHC. Pteroylglutamic acid and *p*-aminobenzoic acid were both also without effect, as were combinations of all the above substances with each other and with cobalamin or EHC. Similar results were obtained by Cross & Woods (1954) in this Laboratory with their extract of alumina-ground organisms. EHC could be replaced, however, by heated extracts of pigeon, pig or rat liver.

Properties and partial purification of extract of heated Escherichia coli

In a study of the conversion of serine into glycine by cell-free extracts of *Clostridium sticklandii* (earlier called *Clostridium* HF) it was found that extracts of heated *Cl. cylindrosporum* had much greater cofactor activity than tetrahydropteroylglutamic acid; the new factor was called CoC (Wright, 1955*a*, *b*, 1956; Wright & Stadtman, 1956). A preparation of CoC kindly provided by Dr Barbara Wright proved to be active for methionine synthesis in the present test system; similarly, crude EHC was active for the conversion of serine into glycine by extracts of *C. sticklandii* (unpublished observations of Dr Barbara Wright). EHC, like CoC (Wright, 1955*a*), was stable in alkali but unstable to acid.

The purification of EHC was therefore attempted by the procedure used for the isolation of CoC. The crude EHC was chromatographed on paper with propanol–pyridine–water as solvent system. The active fraction (R_F 0·33–0·66) was eluted with aq. 10 mM-NH_3 soln. and lyophilized; on the basis of dry weight a fivefold purification was obtained. Over the range tested the effect on methionine synthesis increased linearly with concentration (Fig. 4). The absorption spectrum (after correction for nucleotides present) indicated the presence of a folic acid derivative but no further deductions could be made.

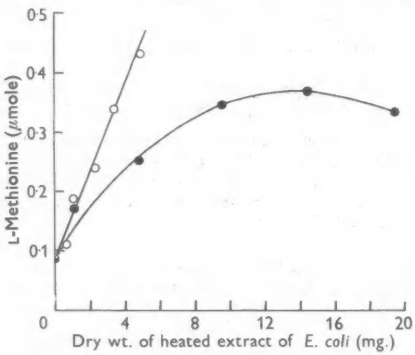

Fig. 4. Effect of extract of heated *E. coli*. Standard reaction mixture containing dialysed enzymic extract (equivalent to 4 mg. of protein) was used with crude (●) or partly purified (○) extract of heated *E. coli* added as indicated. Incubation was for 3 hr. at 37°.

Effect of folic acid conjugates

Wright (1955*b*, 1956) found that CoC could be fractionated into several active compounds, all of them folic acid derivatives. There was evidence that one of them was an *N*-formylpteroyltriglutamic acid, possibly the N^5-compound. A specimen of this material was kindly given by Dr Barbara Wright. Although it increased methionine synthesis under the present conditions it was not as

Table 5. *Effect of folic acid derivatives on methionine synthesis*

Standard reaction mixture containing dialysed enzymic extract equivalent to 3·2 mg. of protein was used, but with the extract of heated *E. coli* (EHC) replaced as shown. A final volume of 1 ml. only was used. Incubation was for 3 hr. at 37°.

Additions		L-Methionine formed
Substance	Concn. (mg./ml.)	(μm-moles)
None	—	60
EHC*	6	185
	(μM)	
N-Formylpteroyltriglutamic acid	6	60
	20	84
	40	100
Pteroyltriglutamic acid	6	64
	120	64

* Acetone-treated extract of heated *E. coli* (20 mg. dry wt./ml.) was used in this experiment (see Methods section).

Table 6. *Incorporation of* [β-^{14}C]*serine into methionine*

Standard reaction mixture (containing dialysed enzymic extract equivalent to 5 mg. of protein) was used except that [β-^{14}C]serine was added to the DL-serine; DL-homocysteine was omitted, as indicated, in one experiment. Incubation was for 3 hr. at 37°.

	Expt. 1	Expt. 2*	
		With homocysteine	Without homocysteine
Specific activity of serine (counts/min./μmole)	$9{\cdot}2 \times 10^3$	$2{\cdot}3 \times 10^4$	$2{\cdot}3 \times 10^4$
L-Methionine formed			
By microbiological assay (μm-moles)	250	280	40
By chromatographic isolation (counts/min.)	1660	5420	880
By chromatographic isolation (μm-moles)†	177	230	40
Activity of methyl*iso*thiouronium salt (counts/min.)	750	3189	—
Activity of residue after distillation of methyl iodide (counts/min.)	45	260	—

* A preparation from heated *C. cylindrosporum* replaced EHC in this experiment.
† Calculated from the activity of serine.

active as EHC on the basis of approximate folic acid content of the latter as judged from the absorption at 260 mμ (Table 5). Pteroyltriglutamic acid (teropterin) had no activity even at three times the concentration of the formyl derivative.

Incorporation of [β-^{14}C]*serine into methionine*

Gibson & Woods (1960) found production of methionine by *E. coli* PA 15 (whole organisms) with serine as C_1-unit donor to be accompanied by the production of approximately equimolar quantities of glycine. It was probable therefore that the methyl group of methionine was derived from the β-C atom of serine; this occurs in animal tissues (Arnstein & Neuberger, 1953; Stekol, 1955). The point was investigated with enzymic extracts of *E. coli* PA 15 by including [β-^{14}C]serine in the standard reaction mixture. Methionine was determined as usual by microbiological assay and, after addition of inactive carrier methionine, by determination of radioactivity after isolation by paper chromatography. The methionine was labelled but the quantity was about 25% smaller than that expected from the microbiological assay (Table 6), probably due to losses during the multiple manipulations with small quantities. The methionine was degraded and over 90% of the total radioactivity recovered (50–60%) was found in the methyl *iso*thiouronium salt (Table 6); the low overall recovery is again probably due to experimental losses.

Fractionation of enzymic extract

The complexity of cofactors required for the methylation of homocysteine suggests that there may be several steps in the reaction; furthermore, the work of Emerson (1950) with *Neurospora* mutants tends to the same conclusion. A simple fractionation of the enzymic extract was therefore attempted to see if two or more defined fractions were required and if there were any evidence for an intermediate.

Table 7. *Fractionation of enzymic extract*

Activity was estimated with the standard reaction mixture.

Fraction	Total protein (mg.)	Total activity (units*)	Specific activity*
Original extract	925	65 000	23
P1	150	2 730	6
P2	11	180	5
P3	145	6 760	16
P4	375	2 500	2
P5	98	1 220	4
P1+P2; P1+P4; P2+P4; P2+P5; P4+P5	—	—	2–7
P3+P4+P5	618	24 500	51†

* For definition of these terms see Methods section.
† Expressed in terms of the protein content of fraction P3 only.

The crude enzymic extract was cooled to 0°, brought to pH 5·2 with N-acetic acid and centrifuged at 16 000 *g* for 10 min. The sediment (P1) was dissolved in minimal volume of 0·2 M-phosphate buffer, pH 7·5.

The supernatant was brought to pH 6·0 with phosphate buffer and precipitated at 0° with increasing concentrations of ammonium sulphate. Fractions were collected successively at 0·35, 0·5, 0·6 and 0·9 saturation (P2–P5 respectively), dissolved in 0·2 M-phosphate buffer and dialysed for 12–14 hr. at 0° against the same buffer.

With the exception of P3 the activity of all the fractions was low compared with that of dialysed crude extract (Table 7). Recombination of pairs of fractions led to no improvement. However, a mixture of fractions P3, P4 and P5 had about 2·5 times the total activity of the sum of the separate

fractions although only about 40% of the original activity was recovered. The relatively high activity of P 3 may therefore be due to some contamination with material precipitated in bulk with P 4 and P 5. The results obtained were not sufficiently clear-cut to offer any hope of detecting and isolating an intermediate.

Formation of glycine from serine

With whole organisms of the present strain Gibson & Woods (1960) found approximately equimolar formation of methionine and glycine from homocysteine and serine, as did also Cross & Woods (1954) with an enzyme preparation made by grinding with alumina. One component of the present enzymic extract might well be therefore an enzyme cleaving serine to glycine, the residual β-C-atom fragment being ultimately donated to homocysteine. A similar enzyme found in animal tissues by Kisliuk & Sakami (1955) has been termed serine hydroxymethylase; it is also present in *C. sticklandii* (Wright & Stadtman, 1956). Gibson & Woods (1960) found some formation of glycine from serine in the absence of homocysteine by suspensions of *E. coli* PA 15.

Table 8. *Production of glycine from serine*

Standard reaction mixture (containing dialysed enzymic extract equivalent to 4·5 mg. of protein) was used except that EHC was increased to 0·4 ml. and DL-homocysteine was omitted as indicated. Incubation was for 3 hr. at 37°.

		Product (μm-moles)	
	Expt.	Glycine*	Methionine
Homocysteine absent	1	150	30
	2	120	35
	3	286	40
Homocysteine present	1	286	185
	2	170	170
	3	260	180

* Control values (serine absent) were 260, 170 and 370 for Expts. 1–3 respectively. These values have been deducted to obtain the figures given.

Table 9. *Cofactor requirements for the formation of glycine from serine*

Standard reaction mixture containing dialysed enzymic extract equivalent to 5 mg. of protein was used but with DL-homocysteine omitted and EHC added as shown; other omissions were as indicated. Incubation was for 3 hr. at 37°.

Vol. of extract of heated *E. coli* (ml.)	Other substances omitted	Glycine formed (μm-moles)*	
		Expt. 1	Expt. 2
0	None	100	—
0·3	None	226	—
0·6	None	300	—
0·5	None	—	280
0·5	ATP + Mg^{2+} ions	—	130
0·5	DPN	—	140
0·5	PO_4^{3-} ions	—	160

* Control values without serine were deducted: these were 160 and 220 for Expts. 1 and 2 respectively.

The products of typical experiments on methionine synthesis by dialysed enzymic extracts both in the presence and in the absence of homocysteine were assayed for glycine (Table 8). The results were irregular but showed definitely the formation of glycine from serine in the absence of homocysteine; in two out of three cases there was some increase of glycine when homocysteine was also present and methionine was formed. It was not expected that the results would have quantitative significance for the following reasons: (*a*) with the method of assay used the control values for glycine (serine absent) were of the same order as the additional glycine formed in the presence of serine; (*b*) a serine deaminase was also present (see beginning of Results section); (*c*) possible variability with different enzyme preparations in the extent of the competition between homocysteine and other endogenous acceptor systems for the C_1-unit fragment released from serine. There was some evidence from isotope experiments that in the absence of homocysteine the other ultimate product was formate.

Cofactor requirements. The amount of glycine formed (homocysteine absent) was much reduced if phosphate, DPN or ATP + Mg^{2+} ions were omitted from the reaction mixture (Table 9). The extract of heated *E. coli* was also necessary; glycine formation was reduced two- to three-fold in its absence (Table 9). The cofactor requirements for glycine formation from serine are thus qualitatively similar to those for methionine formation from homocysteine and serine.

DISCUSSION

The overall methylation of homocysteine by serine requires not only the transfer of the hydroxymethyl group from serine (at the formaldehyde level of oxidation), but also its reduction to a methyl group. Although DPN, which is shown to be required, may serve as intermediate there must be present some hydrogen-donor system. Since in the present work none was deliberately added (or appeared to be required) it must have been derived from the dialysed enzymic extract, from the extract of heated *E. coli* or from one of the substrates. The most probable source would appear to be pyruvate formed from serine by the serine deaminase known to be present; *E. coli* is known to contain a pyruvic-oxidase-enzyme system.

The work of Gibson & Woods (1960) with intact organisms coupled with the results of the present experiments make it reasonably certain that some coenzyme form of folic acid is required for the overall reaction. This would be expected for a C_1-unit-transfer reaction and the extract of heated *E. coli* which was necessary had properties akin to the coenzyme required for glycine formation from serine by *C. sticklandii*; this is certainly a form of folic acid (Wright & Stadtman, 1956; Wright, 1956). Furthermore, a sample of an *N*-formylpteroyltriglutamic acid isolated from *C. cylindrosporum* had activity (though only at high concentrations) in the present system.

In all other C_1-unit-transfer reactions so far examined tetrahydropteroylglutamic acid is active, though it may not be the natural coenzyme (see review by Wright, 1958). Moreover in work with sheep-liver homogenates Nakao & Greenberg (1958) gave evidence that it functions as cofactor in the methylation of homocysteine by serine and formaldehyde. Tetrahydropteroylglutamic acid was, however, completely inactive in the present experiments. It is possible that the bacterial extracts lack some enzyme(s) which convert it into the true coenzyme form or that some other factor is required for its utilization which is not required by the natural coenzyme. More recent work in this Laboratory described briefly by Kisliuk & Woods (1957) and Helleiner, Kisliuk & Woods (1957) has shown that if the organisms are grown originally on a medium containing added cobalamin the extracts of acetone-dried organisms are then able to use tetrahydropteroylglutamic acid as cofactor; furthermore, ultrasonic extracts of organisms from normal growth medium can also do so, but only if cobalamin is added to the extract.

It is clear that serine is degraded to glycine and that the residual β-C-atom of serine becomes the C atom of the methyl group of methionine. The formation of glycine from serine by the enzymic extracts also requires the extract of heated *E. coli* and, on the basis of the present results alone, this might be (though not of necessity) the only function of the coenzyme form of folic acid. Work in this Laboratory (Kisliuk & Woods, 1959), however, has shown that tetrahydropteroylglutamic acid is an effective coenzyme for this reaction under normal test conditions whereas the synthesis of methionine requires the heated extract of the organism. The coenzyme form of folic acid must thus have a function in the acceptance as well as in the generation of the C_1 unit.

SUMMARY

1. Soluble extracts of acetone-dried *Escherichia coli* (an auxotrophic strain requiring serine or glycine for growth) synthesize methionine from homocysteine and serine.

2. After dialysis of the enzymic extract there are requirements (not absolute) for phosphate and magnesium ions, adenosine triphosphate, diphosphopyridine nucleotide and a heated extract of *E. coli*.

3. The heated extract of *E. coli* probably contains a coenzyme form of folic acid. It is not replaced by tetrahydropteroylglutamic acid or its N^5-formyl derivative. It is partially replaced by a *N*-formylpteroyltriglutamic acid extracted from *Clostridium cylindrosporum*.

4. Isotope technique showed that the β-carbon atom of serine became the methyl carbon atom of methionine.

5. The enzymic extracts also degraded serine to glycine; the cofactor requirements were qualitatively similar to those for methionine synthesis.

We are indebted to the Director and Staff (especially Mr F. C. Belton) of the Microbiological Research Establishment, Ministry of Supply, Porton Down, Wilts. for the bulk growth of *E. coli*. Our best thanks are due to Dr M. J. Cross for permission to use the results of his early experiments with extracts of acetone-dried organisms and for other help. We are indebted to Dr Barbara Wright and Dr Thressa Stadtman for information in advance of publication about their method for separating CoC. One of us (J.S.) wishes to thank the British Council and the Centre National de la Recherches Scientifique (France) for fellowships which made this work possible.

REFERENCES

Alexander, B., Landwehr, G. & Seligman, A. M. (1945). *J. biol. Chem.* **160**, 51.

Arnstein, H. R. V. & Neuberger, A. (1953). *Biochem. J.* **55**, 259.

Cross, M. J. & Woods, D. D. (1954). *Biochem. J.* **58**, xvi.

Emerson, S. (1950). *Cold Spr. Harb. Symp. quant. Biol.* **14**, 40.

Gibson, F. (1952). *Abstr. 2nd int. Congr. Biochem., Paris*, p. 80.

Gibson, F. & Woods, D. D. (1952). *Biochem. J.* **51**, v.

Gibson, F. & Woods, D. D. (1960). *Biochem. J.* **74**, 160.

Helleiner, C. W., Kisliuk, R. L. & Woods, D. D. (1957). *J. gen. Microbiol.* **18**, xv.

Kisliuk, R. L. & Sakami, W. (1955). *J. biol. Chem.* **214**, 47.

Kisliuk, R. L. & Woods, D. D. (1957). *J. gen. Microbiol.* **18**, xv.

Kisliuk, R. L. & Woods, D. D. (1959). *Fed. Proc.* **18**, 261.

Kuby, S. A., Noda, L. & Lardy, H. A. (1954). *J. biol. Chem.* **210**, 65.

Lascelles, J., Cross, M. J. & Woods, D. D. (1954). *J. gen. Microbiol.* **10**, 267.

LePage, G. A. (1949). *Biochem. Prep.* **1**, 5.

Nakao, A. & Greenberg, D. M. (1958). *J. biol. Chem.* **230**, 603.

O'Dell, B. L., Vandenbelt, J. M., Bloom, E. S. & Pfiffner, J. J. (1947). *J. Amer. chem. Soc.* **69**, 250.

Shockman, G. D. & Toennies, G. (1954). *Arch. Biochem. Biophys.* **50**, 9.
Stadtman, E. R., Novelli, G. D. & Lipmann, F. (1951). *J. biol. Chem.* **191**, 365.
Stekol, J. A. (1955). In *Amino Acid Metabolism*, p. 509. Ed. by McElroy, W. D. & Glass, H. B. Baltimore: Johns Hopkins Press.
Stickland, L. H. (1951). *J. gen. Microbiol.* **5**, 698.
Szulmajster, J. & Woods, D. D. (1955). *Abstr. 3rd int. Congr. Biochem., Brussels*, p. 44.
Toennies, G. & Shockman, G. D. (1953). *Arch. Biochem. Biophys.* **45**, 447.
Večeřa, M. & Gasparič, J. (1954). *Coll. Trav. chim. Tchécosl.* **19**, 1175.
Vieböck, F. & Brecher, C. (1930). *Ber. dtsch. chem. Ges.* **63**, 3207.
Wright, B. E. (1955*a*). *Biochim. biophys. Acta*, **16**, 165.
Wright, B. E. (1955*b*). *J. Amer. chem. Soc.* **77**, 3930.
Wright, B. E. (1956). *J. biol. Chem.* **219**, 873.
Wright, B. E. (1958). *Proc. 4th int. Congr. Biochem., Vienna*, **11**, 266.
Wright, B. E. & Stadtman, T. C. (1956). *J. biol. Chem.* **219**, 863.

Biochem. J. (1960) **75**, 12

Physical and Chemical Properties of Polysaccharides and Glycoproteins of the Yeast-Cell Wall

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The composition of the yeast-cell wall is known to be approximately glucan 29%, mannan 31%, protein 13%, lipid 8·5% and ash 3% (Northcote & Horne, 1952; Roelofsen, 1953). In addition, numerous investigators have reported the presence of 1–3% of glucosamine, which has been attributed to the occurrence of chitin in the wall (Roelofsen & Hoette, 1951; Houwink & Kreger, 1953; Falcone & Nickerson, 1956; Eddy, 1958). The existence of complexes between the polysaccharide and protein has often been inferred, and recently some evidence for a mannan–protein complex has been obtained (Falcone & Nickerson, 1956; Eddy, 1958). Usually strong alkali has been used to isolate the polysaccharide components and this treatment undoubtedly degrades any complexes between these molecules and protein. We therefore decided in these investigations to use milder extraction procedures. This has resulted in the isolation from the cell wall of several fractions which appear to be glycoproteins. As these fractions also contained glucosamine the distribution of this sugar among the cell-wall components was reinvestigated.

EXPERIMENTAL

Materials. The yeast used was a commercial pressed baker's yeast (Ark Yeast, Distillers Co. Ltd.). Cell walls were prepared according to the method of Shockman, Kolb & Toennies (1957). Each stainless-steel cup (internal measurements 12·5 cm. × 2·5 cm.) contained 20 ml. of a 10% (w/v) suspension of yeast and 20 g. of Ballotini no. 12 glass beads (Chance Bros. Ltd., Birmingham). Breakage was continued for 15 min. in an International refrigerated centrifuge at 1400 rev./min. at 4°. The walls were separated from any unbroken cells and cell contents by differential centrifuging according to Northcote & Horne (1952). The yield of freeze-dried walls was 1 g. from 100 g. of pressed yeast.

Analytical procedures

pH *measurements.* These were made with a Pye pH meter and a glass electrode at room temperature and by standardizing the apparatus with standard buffer tablets, pH values 4·01 and 6·99 (Burroughs Wellcome and Co.).

Boundary electrophoresis. The samples were examined as approximately 1·5% solutions in 0·05M-sodium borate buffer, pH 9·2, 0·1M-K_2HPO_4, pH 9·3, or 0·05M-sodium borate–8M-urea, pH 9·2. The Perkin–Elmer model 38 Tiselius electrophoresis apparatus was used.

Diffusion measurements. These measurements were made over a period of 3 days on 0·6% solutions in 0·1M-KH_2PO_4, pH 4·7. The Tiselius electrophoresis apparatus was used.

Sedimentation measurements. The samples were dissolved in 0·1M-KH_2PO_4 (0·05–2·5%) and sedimented at $2{\cdot}69 \times 10^5 g$ for 2·5 hr. in the Spinco Analytical ultracentrifuge model E (Specialized Instruments Co., Belmont, Calif., U.S.A.). Pictures were taken every 16 min.

Total nitrogen and phosphorus. The micro-Kjeldahl digestion method of Chibnall, Rees & Williams (1943) was used, followed by distillation and titration. Phosphorus was determined according to the method of Fiske & Subbarow (1925).

Neutral sugars. Samples were hydrolysed in 2N-H_2SO_4 and the sugars were identified chromatographically (Northcote, Goulding & Horne, 1958).

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Amino acids. Free amino acids were not present before acid hydrolysis. The samples (0·5–3·0 mg.) were hydrolysed in 6N-HCl (0·5 ml.) for 16 hr. at 105° and evaporated to dryness under reduced pressure in a desiccator containing H_2SO_4 and NaOH. The residue was redissolved in a small volume of water and applied as a spot to Whatman no. 52 filter paper. The amino acids were separated by electrophoresis in 2·5% formic acid (pH 2·1) in a field strength of approx. 130 v/cm. for 25 min. The paper was then dried and developed chromatographically in a direction at right angles to the electric field with butanol–acetic acid–water (3:1:1, by vol.). The amino acids were detected by spraying with 0·2% ninhydrin in 96% ethanol. On separate papers, histidine was detected by the Pauly reagent and arginine by the Sakaguchi reagent (Jepson & Smith, 1953). Cysteine was detected as cysteic acid after oxidation of the samples with performic acid (Sanger, 1949). Tryptophan was determined in the unhydrolysed samples by procedure B of Spies & Chambers (1948).

Amino sugar. Glucosamine was detected as a ninhydrin-coloured spot on the papers used for analysis of the amino acids. Its position relative to the amino acids is shown on Fig. 1. It had an electrophoretic mobility corresponding to alanine and moved chromatographically similarly to glycine. This spot also reduced alkaline silver nitrate (Trevelyan, Procter & Harrison, 1950) and gave a positive reaction with the Elson–Morgan reagent (Partridge, 1948). The hexosamine was further identified as glucosamine after separation electrophoretically by chromatography in pyridine–ethyl acetate–water (1:2:2, by vol.) when glucosamine only was detected and after oxidation by ninhydrin (Stoffyn & Jeanloz, 1954) when only arabinose was detected. In addition, the compound was found to have the same mobility as glucosamine upon electrophoresis in pyridine–acetic acid–water buffer, pH 3·5 (1:10:89, by vol.), and pH 6·5 (10:0·3:90, by vol.). The procedures do not distinguish glucosamine from mannosamine.

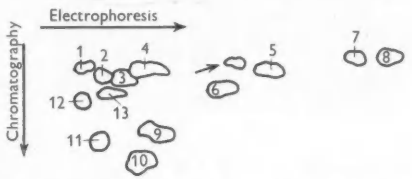

Fig. 1. Separation of amino acids and glucosamine in an acid hydrolysate of fraction *B*. The arrow indicates the glucosamine. Amino acids: 1, aspartic acid; 2, glutamic acid; 3, threonine; 4, serine; 5, glycine; 6, alanine; 7, histidine; 8, lysine; 9, valine; 10, leucine/isoleucine; 11, phenylalanine; 12, tyrosine, 13, methionine.

The glucosamine was quantitatively determined after hydrolysis of the samples in 4N-HCl for 12 hr. at 100° by the Elson–Morgan reaction (Blix, 1948), with both glucosamine and chitin as standards. The chromogen formed had the correct spectrum for glucosamine and no colour developed in the absence of acetylacetone. No free glucosamine was present in any of the samples before acid hydrolysis.

N-Terminal amino acid. The samples (50–200 mg.) were dissolved in 8M-urea containing 1% of $NaHCO_3$, treated with fluorodinitrobenzene, and the dinitrophenyl (DNP)-amino acids were determined according to Fraenkel-Conrat, Harris & Levy (1955).

RESULTS

Fractionation of the cell walls

The cell walls were extracted with anhydrous ethylenediamine at 37° (Fig. 2). The mixture was shaken at intervals of 30 min. to resuspend the walls, which after a period of 6 hr. became gelatinous and settled very slowly. During the remainder of the extraction time the mixture was shaken only about three or four times a day and after 3 days it was divided into three fractions as indicated in Fig. 2. The chemical characteristics of these fractions are shown in Table 1.

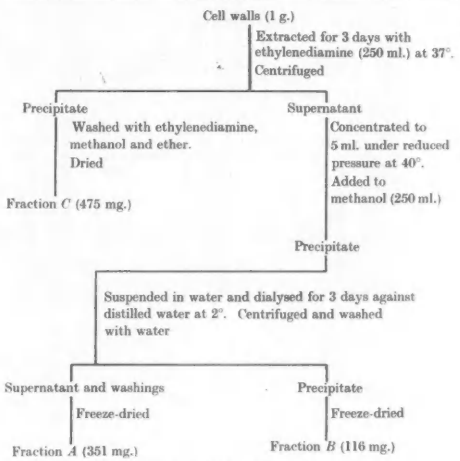
Cell walls (1 g.)
Extracted for 3 days with ethylenediamine (250 ml.) at 37°. Centrifuged
Precipitate
Washed with ethylenediamine, methanol and ether. Dried
Fraction C (475 mg.)
Supernatant
Concentrated to 5 ml. under reduced pressure at 40°. Added to methanol (250 ml.)
Precipitate
Suspended in water and dialysed for 3 days against distilled water at 2°. Centrifuged and washed with water
Supernatant and washings
Freeze-dried
Fraction A (351 mg.)
Precipitate
Freeze-dried
Fraction B (116 mg.)

Fig. 2. Fractionation of the yeast-cell wall by ethylenediamine.

Table 1. *Chemical characteristics of the cell-wall fractions*

The protein was calculated from the N content after correcting for the amount of glucosamine present.

Fraction	Neutral sugars	N (%)	Glucosamine (%)	Protein (%)	Phosphorus (%)
A	Mannose	2·0	1·7	11·6	0·30
B	Mannose, glucose	1·0	0·8	5·9	0·12
C	Mannose, glucose	2·7	2·0	15·9	—
Mannan	Mannose	0·35	1·4	1·5	0·30
Glucan	Glucose	0·6	6·0	0·8	0·008

Cell-wall fraction A. This material gave a positive reaction for protein (Lowry, Rosebrough, Farr & Randall, 1951) and contained the following amino acids: aspartic acid, glutamic acid, threonine, serine, glycine, alanine, valine, leucine or isoleucine or both, proline, phenylalanine, tyrosine, tryptophan, cysteine, lysine and histidine. The only DNP-amino acids detectable were alanine and glycine (approx. 0·1 μmole of each/40 mg.).

The sedimentation diagram of fraction *A* shows two components, the relative amounts of which depended upon the time of treatment (24 or 72 hr.) of the cell walls with ethylenediamine (Fig. 3*a*, *b*). After extraction for 3 days the faster-moving component is considerably reduced in amount (Fig. 3*b*). All the data reported refer to the material prepared by extraction for 3 days. The sedimentation and diffusion constants are shown in Table 2. The molecular weight calculated for the slower (major) component is probably lower than its true molecular weight because of the heterogeneity of the material.

When fraction *A* was subjected to electrophoresis in borate buffer only one peak was observed with a slight asymmetry in the trailing edge (Fig. 4, Table 2). Essentially the same picture was obtained in borate–urea buffer. On electrophoresis in phosphate buffer a much smaller mobility was found (Table 2) but again only one peak was evident.

Cell-wall fraction B. This fraction also gave a positive test for protein and contained the same

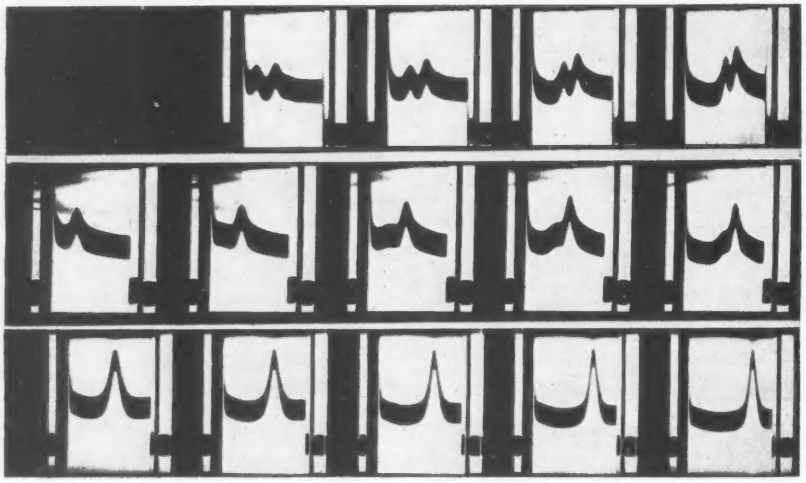

Fig. 3. Sedimentation patterns of yeast-cell-wall fractions dissolved in 0·1 M-KH_2PO_4, pH 4·7. (*a*) Fraction *A* (15 mg./ml.) prepared by extraction of cell walls with ethylenediamine for 24 hr.; (*b*) fraction *A* (15 mg./ml.) prepared by extraction of cell walls with ethylenediamine for 72 hr. (*c*) Mannan (16·4 mg./ml.). Centrifugal force was $2{\cdot}69 \times 10^5$ *g* and the temperature 21°. Photographs were taken at 16 min. intervals. Migration is from right to left.

Table 2. *Physical characteristics of cell-wall fraction A and mannan*

Values for S_{20} and D_{20} were determined at a concentration of 6 mg./ml. in 0·1 M-KH_2PO_4 and the molecular weights were calculated from the equation: mol. wt. $= \dfrac{RTS}{D(1-\bar{v}\rho)}$.

Fraction	$10^{-13} \times$ Sedimentation constant (S_{20}) (cm. g.$^{-1}$ sec.$^{-1}$)	$10^{-7} \times$ Diffusion constant (D_{20}) (cm.^{2} sec.$^{-1}$)	Mol.wt.	$10^{-5} \times$ Electrophoretic mobility (cm.^{2} V^{-1} sec.$^{-1}$)	
				Borate buffer, pH 9·2	Phosphate buffer pH 9·3
Mannan	3·6	5·09	$5{\cdot}9 \times 10^4$	8·8	2·26
A Major peak	4·2	3·50	$9{\cdot}8 \times 10^4$	6·0	1·55
A Minor peak	6·7	—	—	—	—

amino acids as fraction *A*. Again only DNP-alanine and DNP-glycine were obtained and in approximately the same yield as with fraction *A*. Although this fraction was insoluble in water it dissolved in 8 M-urea to give an opalescent solution from which it was quantitatively precipitated upon dialysis against water. The electrophoretic diagram obtained in borate–urea buffer showed the presence of only one component (mobility, $5{\cdot}7 \times 10^{-5}$ cm.^{2} v^{-1} sec.$^{-1}$).

Cell-wall fraction C. Microscopically this fraction resembled the original form of the cell wall but was greatly swollen. When sectioned and examined with the electron microscope (Northcote *et al.* 1958) two distinct membranes were no longer discernible.

Mannan

This polysaccharide ($[\alpha]_D^{17}$ 88·5° in water (*l*, 2; *c*, 1·1) was prepared from whole yeast by the method of Haworth, Heath & Peat (1941). It gave no colour with iodine. It had a partial specific volume ($\bar{v}$) of 0·65. Although it gave a negative test for protein (Lowry *et al.* 1951) it was found to contain the same amino acids as cell-wall fraction *A*, and again DNP-alanine and -glycine were detected (approx. 0·1 μmole/500 mg.). The electrophoresis diagram of mannan in borate buffer showed only one symmetrical peak (Northcote, 1954). In phosphate buffer again only one peak was observed but it had a much smaller mobility (Table 2). The leading edge of the peak diffused rapidly. This is characteristic of polysaccharides when a buffer not containing borate is used (Northcote, 1954). The sedimentation diagram showed one symmetrical peak (Fig. 3*c*). Other chemical and physical characteristics of this material are shown in Fig. 5 and Tables 1 and 2.

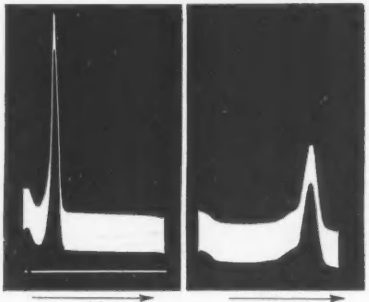

Fig. 4. Boundary electrophoresis of fraction *A* (15 mg./ml.) in 0·05 M-borate buffer, pH 9·2. (*a*) Descending limb, 40 min.; (*b*) descending limb, 150 min.

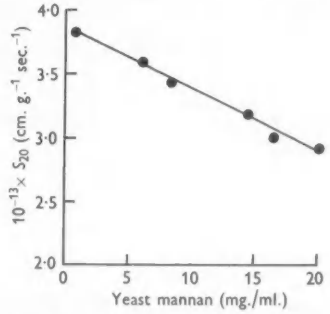

Fig. 5. Variation of sedimentation constant (S_{20}) with concentration of yeast mannan in 0·1 M-KH_2PO_4.

Glucan

This insoluble polysaccharide was prepared from whole yeast by the method of Bell & Northcote (1950). The chemical characteristics of this material are shown in Table 1. It gave a negative test for protein (Lowry *et al.* 1951) but contained all the amino acids found in the other materials, except that tyrosine, phenylalanine and proline were not detected.

Tests for chitin

Glucan (200 mg.) was boiled with 2 % HCl for 1 hr. and then with 3 % NaOH for 15 min. This treatment dissolved 80 % of the glucosamine originally present in the polysaccharide. The residue was heated at 160° for 15 min. in 60 % KOH to prepare chitosan (Roelofsen & Hoette, 1951), but the small residue which remained did not stain with iodine.

Cell-wall fraction *C* (200 mg.) was also treated in the manner described above, when 85 % of the glucosamine originally present dissolved in the dilute acid and alkali. There was no residue left after treatment with 60 % KOH.

Chitin (100 mg.) prepared from crab shells (Purchase & Braun, 1946) was extracted for 3 days at 37° with ethylenediamine (150 ml.). No glucosamine could be detected in the solution and chitin was recovered quantitatively. The chitin (10 mg.) was then treated with dilute acid and alkali in the manner described above. The insoluble residue was found to contain 90 % of the glucosamine of the chitin.

DISCUSSION

Of the total glucosamine in the cell wall, fraction *A* contains 36 %, fraction *B*, 6 % and fraction *C*, 58 %. Fraction *A* is soluble in both ethylenediamine and water and fraction *B* is soluble in ethylenediamine. As chitin is insoluble in both these solvents, the glusamine in fractions *A* and *B*

cannot be present in the form of chitin. When fraction *C* is treated with dilute acid and alkali, 85% of the glucosamine is dissolved and therefore a maximum of 15% of this fraction (9% of the total glucosamine of the cell wall) has the solubility properties of chitin. Similarly, the presence of glucosamine in the soluble mannan and the solubility characteristics of most of the glucosamine in the glucan again indicate that only a small percentage of the total glucosamine in the yeast-cell wall could be chitin. This residual insoluble material could possibly account for the chitin detected by the X-ray-diffraction studies of Houwink & Kreger (1953).

The presence of bound glucosamine in the glucan and mannan which have been prepared by standard procedures raises the question whether the amino sugar occurs in these materials as a separate polysaccharide or as part of the glycosidic chains of the glucan and mannan. In either case it must be considered in chemical structural studies of these compounds and this is especially important with glucan, in which the content of glucosamine is as high as 6%.

The 16 amino acids found in fraction *A* are present in peptide form and as only two *N*-terminal amino acids are found it is probable that the protein portion of the molecule is a specific compound. The low recoveries of DNP-amino acids were probably due to unavoidable losses in the acid hydrolysis because of the large amounts of carbohydrate, and do not indicate the true molecular weight of the protein.

The electrophoretic mobility of fraction *A* in borate buffer is of the same order of magnitude as that found for mannan and must therefore be due to the formation of a complex of the carbohydrate portion with the borate ions. However, the mobility is lower than that of mannan, as would be expected if the protein and polysaccharide are firmly bound. Electrophoresis in the presence of 8M-urea and in phosphate buffer showed no evidence for a separate protein peak.

Fraction *A* would seem to represent a mannan–protein complex similar to those described by Falcone & Nickerson (1956) and Eddy (1958). As the molecular weight of even the slower-sedimenting component of fraction *A* is considerably greater than that of mannan, it may be most accurate to consider the components of fraction *A* and mannan as a sequence of degradation products derived from a much larger mannan–protein complex present in the original cell wall.

Fraction *B* has very similar electrophoretic and chemical characteristics to those of fraction *A* but it contains glucose in addition to mannose and therefore might represent a protein–carbohydrate complex in which glucan and mannan are linked together by protein. As the *N*-terminal amino acids found in this fraction are the same as those found in fraction *A* both may contain the same proteins. If fraction *B* is a mannan–glucan–protein complex, it may be a much closer representation than fraction *A* of the true chemical structure of the yeast-cell wall.

It is noteworthy that all the fractions, including mannan and glucan, contain glucosamine. This sugar might serve as the connecting link between the protein and polysaccharide components of the complexes.

SUMMARY

1. The isolated yeast-cell wall has been separated into three fractions: fraction *A*, soluble in ethylenediamine and water; fraction *B*, soluble in ethylenediamine and insoluble in water; fraction *C*, insoluble in both ethylenediamine and water.

2. The physical and chemical characteristics of these fractions have been determined, from which it is concluded that fraction *A* is a mannan–protein complex and fraction *B* may possibly be a glucan–mannan–protein complex. All three fractions contain glucosamine, and it is suggested that this amino sugar might provide the link between the protein and carbohydrate.

3. It is estimated that no more than approximately 9% of the total glucosamine of the cell wall could be present as chitin.

4. The molecular weight of yeast mannan was found to be $5{\cdot}9 \times 10^4$. It contained 1·4% of glucosamine and 1·5% of protein.

5. Yeast glucan was found to contain 6·0% of glucosamine and 0·8% of protein.

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REFERENCES

Bell, D. J. & Northcote, D. H. (1950). *J. chem. Soc.* p. 1944.
Blix, G. (1948). *Acta chem. scand.* **2**, 467.
Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 354.
Eddy, A. A. (1958). *Proc. Roy. Soc.* B, **149**, 425.
Falcone, G. & Nickerson, W. J. (1956). *Science*, **124**, 272.
Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
Fraenkel-Conrat, H., Harris, J. I. & Levy, A. L. (1955). *Meth. biochem. Anal.* **2**, 359.
Haworth, W. N., Heath, R. L. & Peat, S. (1941). *J. chem. Soc.* p. 833.
Houwink, A. L. & Kreger, D. R. (1953). *Leeuwenhoek ned. Tijdschr.* **19**, 1.
Jepson, J. P. & Smith, I. (1953). *Nature, Lond.*, **172**, 1100.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
Northcote, D. H. (1954). *Biochem. J.* **58**, 353.
Northcote, D. H., Goulding, K. J. & Horne, R. W. (1958). *Biochem. J.* **70**, 391.
Northcote, D. H. & Horne, R. W. (1952). *Biochem. J.* **51**, 232.
Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
Purchase, E. R. & Braun, C. E. (1946). *Org. Synth.* **26**, 36.
Roelofsen, P. A. (1953). *Biochim. biophys. Acta*, **10**, 477.
Roelofsen, P. A. & Hoette, I. (1951). *Leeuwenhoek ned. Tijdschr.* **17**, 27.
Sanger, F. (1949). *Biochem. J.* **44**, 126.
Shockman, G. D., Kolb, J. J. & Toennies, G. (1957). *Biochim. biophys. Acta*, **24**, 203.
Spies, J. R. & Chambers, D. C. (1948). *Analyt. Chem.* **20**, 30.
Stoffyn, P. J. & Jeanloz, R. W. (1954). *Arch. Biochem. Biophys.* **52**, 373.
Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950). *Nature, Lond.*, **166**, 444.

Biochem. J. (1960) **75**, 17

The Frequencies of Certain Sequences of Nucleotides in Deoxyribonucleic Acid

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The degradation of deoxyribonucleic acid by 66% (v/v) formic acid containing 2% of diphenylamine, at room temperature (Burton, 1956; Burton & Petersen, 1957), appears to be a valuable reaction for the study of certain sequences in the polydeoxyribonucleotide chains. In this paper we describe the properties of the reaction and a simple chromatographic method by which the frequencies of a considerable number of pyrimidine sequences in deoxyribonucleic acid can be measured on, if necessary, as little as 10–20 mg. of the nucleic acid.

MATERIALS AND METHODS

Deoxyribonucleic acid. This was prepared from calf thymus by extracting the nucleoprotein with 10% (w/v) NaCl and subsequent treatment with sodium dodecyl sulphate (Chargaff, 1955). A sample was freeze-dried to constant weight and found to contain 6·3% of P. Base analysis by hydrolysis in 7·5N-perchloric acid and paper chromatography in propan-2-ol–conc. HCl–water (65:20:10, by vol.; Wyatt, 1955) gave the following molar proportions (mean ± S.E.M.); adenine 28 ± 0·3, guanine 20·9 ± 0·4, cytosine 21·4 ± 0·4, thymine 28·3 ± 0·6, 5-methylcytosine approx. 1. Between 90 and 95 moles of bases were recovered/100 g.atoms of P. These values were obtained from a total of 10 analyses on two hydrolysates.

Apurinic acid. This was prepared from deoxyribonucleic acid (DNA) according to Tamm, Hodes & Chargaff (1952).

Diphenylamine. This was either analytical-reagent grade or a less-pure grade that was recrystallized several times from aqueous ethanol.

Formic acid. Analytical-reagent grade (98–100%) was used.

Calcium bis(p-nitrophenyl)phosphate. This was prepared according to Privat de Garilhe & Laskowski (1955).

Ion-exchange resins. Dowex-1 was nominally 8 or 10% cross-linked and 200–400 mesh. The finer particles were removed by decantation after suspension in water, the resin was packed into a column and washed with 2N-NaOH, water and 3N-HCl until the extinction coefficient of the eluate was less than 0·01 at 260 mμ. Formate and acetate forms were prepared from the chloride form as described by Sinsheimer (1954). Dowex-50 (200–400 mesh) was cleaned according to Moore & Stein (1951). The Dowex-1 was kept clean until required by slowly percolating a solution of the corresponding ammonium salt (0·01M) through the resin.

Paper for two-dimensional chromatography. Sheets of Whatman no. 1 paper for chromatography (47 cm. × 53 cm.) were serrated along the short edge and washed for about 15 hr. with N-HCl by allowing the acid to run along the paper and off the serrated edge in a tank for descending chromatography. They were then similarly washed with water for about 30 hr. and dried in air at room temperature.

Snake-venom phosphodiesterase. This was prepared from *Crotalus adamanteus* venom (Ross Allen's Reptile Institute, Silver Springs, Fla., U.S.A.) by the method of Cohn, Volkin & Khym (1957), including a second acetone fractionation. An increase of 0·13 in the extinction coefficient at 440 mμ was given by 0·1 ml. of the preparation under the conditions of Cohn *et al.* (1957).

Spleen phosphodiesterase. This was prepared according to Heppel & Hilmoe (1955) and dialysed against 0·1M-NaCl and then against water to remove pyrophosphate. The final preparation contained 30 units of activity/ml. (units as defined by Heppel & Hilmoe).

Prostatic phosphomonoesterase. Hypertrophic human prostate glands were dissected free of connective tissue and

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fat and the enzyme was then extracted and purified by salt fractionation. For one preparation purified by the method of Markham (1955), the ratio of monoesterase to diesterase activity was 35. Preparations by two other methods (Kerr & Chernigoy, 1957; Davidson & Fishman, 1959) gave a ratio of 1700. Each of these preparations was further purified by chromatography on 2% cross-linked Dowex-50, adsorption of the enzyme at pH 5·2 or 5·5 and elution with buffer at pH 5·7 (Boman, 1955; Privat de Garilhe, Cunningham, Laurila & Laskowski, 1957). The active fractions were dialysed against water at 4° until free of phosphate and concentrated by freeze-drying to give solutions with about 700 units of monoesterase/ml. Before dialysis, sufficient stabilizing protein (egg albumin) was added to give a final concentration of 0·5%. The ratio of monoesterase to diesterase activity was then $4–7 \times 10^4$.

Monoesterase activity was measured by incubating a sample of enzyme at 37° in a total volume of 3·2 ml. of solution containing 1·6 mM-disodium *p*-nitrophenylphosphate and buffered at pH 5·6 with 6·2 mM-acetic acid–56 mM-sodium acetate. After a suitable time the reaction was stopped and the colour of the free *p*-nitrophenol was developed by addition of 0·2 ml. of aq. 2N-NH_3 soln. One unit of activity is defined as the amount of enzyme causing an increase of unity in the extinction coefficient at 400 mμ in 1 min., compared with a control sample containing no enzyme. Diesterase activity was measured similarly but with the calcium bis-(*p*-nitrophenyl)phosphate as substrate and stopping the reaction with aq. 2N-NH_3 soln. containing 0·1M-tetrasodium ethylenediaminetetra-acetate (EDTA). The EDTA was included to prevent the precipitation of calcium phosphate.

Inorganic phosphate. The procedure of Berenblum & Chain (1938) as modified by Bartley (1953) has been used for the reaction mixtures containing amine and formic acid. For samples that had been degraded and extracted with ether, the more convenient method of Chen, Toribara & Warner (1956) was used with a total volume of 4 ml. Both methods gave the same values on these mixtures. For the eluate from paper chromatograms, the method of Chen *et al.* was scaled to a total volume of 0·4 ml.

Total phosphorus. Samples containing about 1 μmole of phosphate were measured into a 150 mm. × 14 mm. Pyrex test tube and a small alundum chip was added. The tubes were supported vertically in a tin-plated can and dried by an infrared lamp. The ashing fluid was conc. H_2SO_4–60% perchloric acid (3:2, v/v) saturated with ammonium molybdate as a catalyst; 0·5 ml. of this reagent was added to each tube and the contents were ashed by gentle boiling, which was continued for 3–5 min. after the mixture became clear. When cool, the contents of the tubes were diluted to 25 ml. and phosphate was measured on 2 ml. by the method of Chen *et al.* (1956) with a total volume of 4 ml.

To measure the P content of spots on paper chromatograms a suitable portion of the eluate containing 1–15 μmoles of phosphate was measured into a 90 mm. × 7 mm. hard-glass test tube and dried under an infrared lamp. A mixture of 3 vol. of conc. H_2SO_4 and 2 vol. of 60% perchloric acid was saturated with ammonium molybdate and 12·5 μl. was measured into each tube and allowed to drain to the bottom. The tubes were heated in an oil bath at about 180° for 4 min. and cooled. The colorimetric reagent was prepared just before use by mixing 15 vol. of 17 mM-EDTA with 2 vol. of 10% ascorbic acid and 2 vol. of 5% ammonium molybdate. Of this, 0·38 ml. was added to each tube and immediately mixed with its contents. The samples were incubated at 37° for 2–3 hr. and the extinction was measured at 820 mμ against that of a reagent blank in cells of 0·4 ml. capacity and 1 cm. light path. The reading was proportional to the phosphate content, 10 μm-moles giving a reading of about 0·77. The EDTA was included because it prevents the development of a spurious blue colour.

Monoesterified phosphate. The solution containing about 0·4 mM-phosphate was made 0·01–0·02M with respect to EDTA, adjusted to pH 5·6 and incubated at 37° with 0·05–0·1 vol. of phosphomonoesterase until dephosphorylation was complete (usually overnight). Protein was precipitated by 0·5M-perchloric acid and inorganic phosphate was estimated on the solution according to Chen *et al.* (1956).

Spectrophotometric measurements. These were made with a Beckman model DU quartz spectrophotometer in cells of 1 cm. light path and with correction for cell blanks. The readings obtained with this instrument were identical with those obtained on two similar ones.

Desalting. Several methods were used for removing salt from the fractions obtained after ion-exchange chromatography. Formic acid and ammonium formate were removed by sublimation on a freeze-drying apparatus at about 40°. Hydrochloric acid was removed from most of the fractions by adsorbing the nucleotides onto Nuchar charcoal (West Virginia Pulp and Paper Co., New York 17, N.Y., U.S.A.). The charcoal was then washed with water and the nucleotides were eluted with 50% ethanol containing 2% (v/v) of conc. aq. NH_3 soln. Ammonia and ethanol were then removed in a rotary evaporator. The trinucleotide products were freed of HCl by dialysing against distilled water for about 2 hr. in dialysis tubing that had been purified by heating in several changes of water (Hughes & Klotz, 1956). The nucleotides themselves diffused only very slowly.

Nomenclature of pyrimidine nucleotides

The system of abbreviations described by Privat de Garilhe & Laskowski (1956) has been used with the slight modification that, since all the products described here are deoxyribose derivatives, the symbol (*d*) is omitted. C, T and M represent deoxycytidine, thymidine and 5-methyldeoxycytidine respectively. p represents orthophosphate esterified with these nucleosides. If p appears on the left of the nucleoside symbol it represents a 5′-phosphate, if on the right a 3′-phosphate. When p appears between two capital letters it represents the 3′:5′-phosphodiester bridge linking the two nucleosides.

EXPERIMENTAL AND RESULTS

Effects of amines on the liberation of inorganic phosphate from deoxyribonucleic acid and apurinic acid

Table 1 shows the effect of a number of amines on the liberation of inorganic phosphate from DNA in 66% (v/v) formic acid. No aliphatic amine caused any detectable release of phosphate, but all the primary and secondary aromatic amines tested

were effective except for 2:4-dinitroaniline and 4-nitrodiphenylamine, which were very poorly soluble in the reaction mixture. Only very small amounts of phosphate (0·3 and 1·4 % respectively) were released by the slightly soluble 2-nitro and 2:4-dinitro derivatives of diphenylamine. Triphenylamine was not effective. Of the amines tested, diphenylamine was the most effective and *p*-nitroaniline was almost as good, followed by *p*-aminoaniline and 1-naphthylamine.

Table 1. *Inorganic phosphate released from calf-thymus deoxyribonucleic acid incubated with various amines in acid solution*

Two volumes of a 3 % solution of the amine were added to 1 vol. of aq. DNA (0·6 mg./ml.). Inorganic phosphate was measured after 6·5 hr. at 30°. No phosphate was released with formic acid alone or with benzylamine, dibenzylamine, ethanolamine or triethylamine.

Amine	Inorganic P (% of DNA P)
Aniline	10·5
o-Nitroaniline	7·1
m-Nitroaniline	13
p-Nitroaniline	23
o-Aminoaniline	3·4
m-Aminoaniline	0·5
p-Aminoaniline	18·4
o-Hydroxyaniline	8·5
m-Hydroxyaniline	4·3
p-Chloroaniline	8
p-Methoxyaniline	6·4
Diphenylamine	25
1-Naphthylamine	16·7
2-Naphthylamine	11·7
Triphenylamine	0

The release of phosphate from apurinic acid is more rapid than it is from DNA, but again diphenylamine was the most effective of the amines that were tested. Table 2 shows the action on apurinic acid of diphenylamine and of some other amines.

The reaction is complicated by a side reaction in which the amines are converted into inactive derivatives by the formic acid. This was not particularly serious with diphenylamine because a solution of 3 % of diphenylamine in formic acid retained about one-third of its original activity when it was stored at room temperature for 4 days. Except in this one experiment, the amines were dissolved in the formic acid shortly before use.

Apart from aqueous acetic acid, no satisfactory alternative solvent has yet been found. As shown in Table 2, the degradation is much slower if half of the formic acid is replaced by *N*-dimethylformamide. Small amounts of phosphate were liberated in various mixtures of dilute HCl and dimethylformamide.

Fig. 1 shows the appearance of inorganic phosphate from calf-thymus DNA. The reaction is complete after about 16 hr. and the amount of inorganic phosphate then remains constant at 27·0–28·5 % of the total phosphate. The lability of the linkages between deoxyribose and the purines and the relative stability of those involving the pyrimidines suggest that the inorganic phosphate arises from phosphodiester groups joining two purine nucleosides (Burton, 1956). The pyrimidine nucleosides are expected to remain as nucleotides of

Table 2. *Release of inorganic orthophosphate from apurinic acid*

To an aqueous solution of apurinic acid (8·7 m-moles of P/l.) was added 2 vol. of a 3 % solution of the amine in either (A) formic acid or (B) a mixture of equal volumes of formic acid and *N*-dimethylformamide. *N*-Phenyl-2-naphthylamine was not completely dissolved in mixture (A). Samples containing *p*-aminodiphenylamine had to be extracted with ether before estimation of the inorganic phosphate. No detectable phosphate was liberated when no amine was added.

(A) Formic acid

Time (hr.)	Inorganic P (% of total)			
	Diphenylamine	*p*-Nitroaniline	*N*-Phenyl-2-naphthylamine	*p*-Amino-diphenylamine
0·25	25	14	—	—
0·33	25·5	—	9·3	18
0·50	25·5	20·5	—	—
1	27	—	21	23
5	27	25	26	22
24	27·5	29	—	—

(B) Formic acid and *N*-dimethylformamide

Time (hr.)	Inorganic P (% of total)		
	Diphenylamine	*N*-Phenyl-1-naphthylamine	*N*-Phenyl-2-naphthylamine
0·5	10·5	5	—
1·0	16·2	8·5	—
5	26·5	23	17
24	27·7	26	27

the general structure Py_nP_{n+1} (where Py is a pyrimidine nucleoside and p is a mono- or di-esterified phosphate group). Since the molar amounts of purine and pyrimidine bases in DNA are equal, the amount of diesterified phosphate between adjacent purine nucleosides must equal the amount between the pyrimidine nucleosides. Thus the amount of inorganic phosphate that is released in the degradation by diphenylamine should equal the amount of organic phosphate that remains after the exhaustive treatment of the pyrimidine nucleotide products with a specific phosphomonoesterase. As shown in Table 3, this expectation was fulfilled.

In another experiment, samples were taken after degradation with diphenylamine for 16, 24 and 40 hr. Each sample was then diluted with an equal volume of water and extracted with ether to remove the diphenylamine and most of the formic acid. They were then treated with phosphomonoesterase and the final amount of inorganic phosphate was found to be 72·8, 73·8 and 72·2 % of the total phosphate in the respective samples. Prolonged incubation with the diphenylamine and formic acid does not therefore cause any further increase in the amount of terminal-phosphate groups and so it does not lead to non-specific splitting of interpyrimidine linkages. Further evidence for this was obtained by incubating DNA in 66 % (v/v) formic acid for 24 hr. at 30° and then adding solid diphenylamine and incubating for a further 24 hr. The inorganic phosphate values before and after phosphomonoesterase were 25 % and 72·8 % respectively.

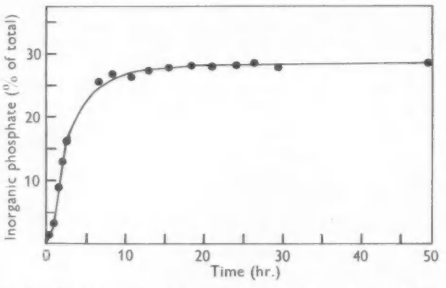

Fig. 1. Release of inorganic phosphate from DNA. Aqueous DNA (0·6 mg./ml.) was incubated at 30° with 2 vol. of a 3 % solution of diphenylamine in formic acid.

Table 3. *Action of prostatic phosphomonoesterase on the products obtained by degrading deoxyribonucleic acid with diphenylamine*

DNA was degraded with diphenylamine in formic acid and extracted with ether. An amount containing 5·6 μ-moles of total P was incubated with 370 units of phosphomonoesterase in 15 ml. of 0·15 M-acetate buffer plus 0·07 M-EDTA, pH 5·6, at 37°. Inorganic phosphate was measured on 1 ml. samples after deproteinizing with perchloric acid.

Time (hr.)	Inorganic phosphate (% of total P)
0	28
2	65·7
3	69
4	72
6	71·2
10	72

Separation of the pyrimidine nucleotide products

Burton & Petersen (1957) described a preliminary separation of the products by chromatography on Dowex-1 chloride, eluting them with HCl. This led to the separation of thymidine and deoxycytidine 3′:5′-diphosphate (pCp and pTp) and the dinucleotide containing cytidine, thymidine and three phosphate groups (CTp_3). Data on these fractions are included in Table 5. Improved separations were, however, obtained by eluting the compounds from Dowex-1 formate by mixtures of ammonium formate and formic acid and such an experiment is here described in detail.

A solution of 1 g. of calf-thymus DNA in 150 ml. of water was incubated for 17 hr. at 30° with 300 ml. of a 3 % (w/v) solution of diphenylamine in formic acid. To remove formic acid and diphenylamine, the mixture was poured into an equal volume of water, extracted five times with about 5 vol. of ether and the aqueous layer was then extracted with ether in a continuous liquid–liquid extraction apparatus for about 4 hr. Solid NaOH was added to the ether in the extraction flask to absorb formic acid. The aqueous phase was made alkaline with ammonia and passed through a column of Dowex-1 formate, 17 cm. × 2·3 cm. diam., which adsorbed the purines and the phosphorus-containing compounds. The free purines were washed from the resin with 10·8 l. of 5 mM-formic acid, and the pyrimidine nucleotides were then eluted with the ammonium formate–formic acid mixtures shown in Fig. 2. Fractions (10 ml.) of the eluate were collected and the extinction coefficients of the fractions were measured at 267 mμ against a blank solution that contained the same concentrations of ammonium formate and formic acid. The linear gradients were obtained as described by Parr (1954). The overall recovery of ultraviolet absorption was 90 %, representing about equal recovery of the purines and pyrimidines of the original digest.

The fractions were pooled into the eight groups shown in Table 4. Most of these were purified by rechromatography on Dowex-1, with either the

chloride form and elution with HCl or the formate form and elution with ammonium formate and 0·05 M-formic acid. The re-adsorption of large volumes for rechromatography was conveniently accomplished by adsorbing the alkaline material on to a short wide column (approx. 4 cm. × 2·5 cm. diam.) and attaching this to the top of a longer column (16–24 cm. × 1 cm. diam.) before beginning the elution. Each purified component was concentrated and freed of salt before further characterization.

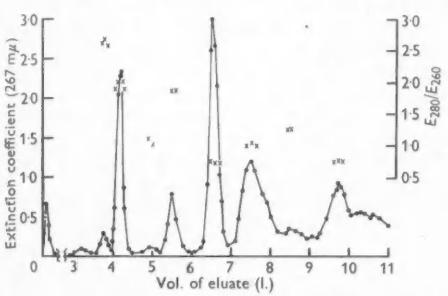

Fig. 2. Separation of pyrimidine nucleotides on Dowex-1 formate. The free purines had previously been washed from the resin with 10·8 l. of 5 mM-formic acid. The pyrimidine nucleotides were eluted with mixtures of formic acid and ammonium formate: 0–0·7 l., 0·01 M-formic acid–0·05 M-ammonium formate; 0·7–2·78 l., 0·01 M-formic acid containing a linear gradient in ammonium formate concentration from 0·06 to 0·25 M; 2·78–2·92 l., 0·01 M-formic acid–0·05 M-ammonium formate; 2·92–12·07 l., 0·1 M-formic acid containing a linear gradient in ammonium formate concentration from 0·06 to 0·56 M in the first 5 l. and from 0·56 to 2·0 M in the remaining 4·15 l. ●, Extinction coefficient at 267 mμ; ×, ratio of extinction at 280 mμ to that at 260 mμ (E_{280}/E_{260}) after adjusting to pH 1·0–2·0. See Tables 4 and 5 for the identities of the components.

Tables 4 and 5 summarize most of the pertinent details of these products and of their rechromatography. Further details are as follows.

Group 1. Adenine and guanine were detected after paper chromatography in propan-2-ol–HCl (Wyatt, 1955). The fraction contained only 0·008 % of the total P applied to the column.

Group 2. Ammonium formate was removed from a portion of this peak by sublimation *in vacuo* and the residue was hydrolysed by heating with 0·1 ml. of 7·5 N-perchloric acid for 1 hr. at 100°. Paper chromatography in propan-2-ol–HCl (Wyatt, 1955) showed the presence of two spots, identified as 5-methylcytosine and cytosine respectively on the basis of their chromatographic position and ultraviolet absorption at 280 and 260 mμ. The ratio of 5-methylcytosine to cytosine was 4·7 to 1.

Group 3. This fraction was eluted as a sharp peak with a constant ratio of E_{280} to E_{260} and in a similar position to that found by Shapiro & Chargaff (1957*b*) for deoxycytidine 3′:5′-diphosphate. The material was accidentally lost, but its identity is confirmed by the fact that the amount obtained from the DNA was closely similar to that which was previously obtained by separation on Dowex-1 chloride (see Table 5).

Table 4. *Details of the rechromatography of pyrimidine nucleotides*

Group	Position in Fig. 2 (l.)	E_{280}/E_{260} (pH 1·0–2·0)	Percentage of u.v. absorption at 267 mμ	Rechromatography: Eluent	Rechromatography: Gradient	Rechromatography: Recovery (%)	Principal constituents
1	0–3·4	—	0·78	—	—	—	Adenine guanine
2	3·4–3·95	2·64	0·52	—	—	—	Mp_2
3	3·95–4·7	1·96	3·0	—	—	—	Cp_2
4	5·1–6·0	1·6	1·7	Formic acid (0·05 M)–ammonium formate (gradient)	0·2–0·4 M in 1 l.	—	C_2p_3
5	6·0–6·95	0·8	2·9	Formic acid (0·05 M)–ammonium formate (gradient)	0·3–0·55 M in 1·5 l.	95	Tp_2
6	6·95–8·4	1·26	5·0	HCl	0·01–0·04 M in 3 l.	*c.* 100	CTp_3 C_2Tp_4
7	8·4–9·2	1·38	2·2	HCl	0·01–0·04 M in 4 l.	*c.* 100	C_2Tp_4
8	9·2–11·1	0·99	7·9	HCl	0·07–0·12 M in 3·5 l.	*c.* 100	$C_2T_2p_5$ T_2p_3

Table 5. *Pyrimidine nucleotide phosphates separated and identified by anion-exchange chromatography*

For evaluation of the amounts, see text. The values for the molar-extinction coefficient at 280 mμ (ϵ_{280}) were determined from the u.v. absorption and the phosphorus content.

Product	Pyrimidine (moles/100 g.atoms of P)	E_{280}/E_{260} (pH 1·0–2·0)	Percentage of total P as end group: Found	Percentage of total P as end group: Expected	$10^{-3}\,\epsilon_{280}$/mole
		Separation on Dowex-1 chloride			
pMp	0·3	—	—	—	—
pCp	3·7	2·0	—	—	14·0
pTp	5·5	0·69	—	—	6·4
CTp_3	5·2	1·25	—	—	—
		Separation on Dowex formate			
pMp	0·5	—	95	100	—
pCp	3·7	1·86	—	—	—
pTp	6·1	0·69	100	100	6·8
pCpCp	1·8	1·9	86	67	27·3
pTpTp	—	0·73	62	67	—
pCpTp	3·2	1·23	67	67	19·5
pTpCp	2·2				
C_2Tp_4	1·23 (group 6)	1·46	60	50	—
	2·03 (group 7)	1·46 (pCpTpCp)	52	50	—
		1·46 (pTpCpCp)	45	50	—
		1·46 (pCpCpTp)	60	50	—

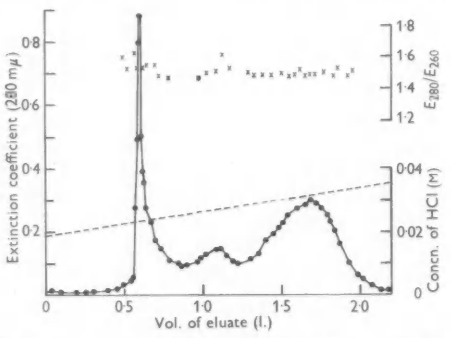

Fig. 3. Separation of C_2Tp_4 isomers on Dowex-2 chloride by gradient elution with HCl. Rechromatography of group 7. ●, Extinction coefficient at 280 mμ; ×, E_{280}/E_{260}; - - -, concentration of HCl. The three isomers are eluted in the order pCpTpCp, pTpCpCp and pCpCpTp.

Group 4. On rechromatography it gave one sharp peak with a small earlier peak which was assumed to be deoxycytidylic acid or pCp arising by spontaneous decomposition of the main material.

Group 5. On rechromatography it gave one sharp peak with a small earlier peak assumed to be thymidylic acid that had been formed by decomposition of pTp.

Group 6. This gave three peaks on rechromatography. The first (at 0·5–0·8 l.) was a mixture that was not resolved on further chromatography on Dowex-1 formate. The second peak, at 1·4–2·0 l., was identified as C_2Tp_4 and the third peak, at 2·1–2·7 l., was identified as CTp_3.

Although Cohn & Volkin (1957) have separated the two isomers of CTp_3 by chromatography on Dowex-1 chloride, we were not able to obtain a separation under similar conditions. The proportions of the two isomers were determined by the method of Privat de Garilhe *et al.* (1957), in which the material is dephosphorylated by phosphomonoesterase and the remaining CpT and TpC compounds are separated by paper chromatography.

Group 7. The principal component of this group (C_2Tp_4) became partially separated into its three isomers when chromatographed on Dowex-1 chloride (Fig. 3).

The identity of each isomer was established by degrading the dephosphorylated material with either spleen or snake-venom diesterases. These two enzymes catalyse the hydrolysis of the 5′- and 3′-phosphoester bonds respectively. The three C_2Tp_2 isomers obtained after phosphomonoesterase treatment would therefore yield the following products when incubated with these enzymes:

	snake venom		spleen diesterase	
T + pC + pC	←	TpCpC	→	Tp + Cp + C
C + pT + pC	←	CpTpC	→	Cp + Tp + C
C + pC + pT	←	CpCpT	→	Cp + Cp + T

Material from each peak was treated with phosphomonesterase and heated at 100° for 15 min. to inactivate the enzyme. The solutions were centrifuged and concentrated by a current of air at 37°. A portion was adjusted to pH 8–9 by adding aq. N-NH_3 soln. and incubated at 37° for 6 hr. with 0·1 ml. of the snake-venom enzyme in 1·5–2·0 ml. of solution containing 0·01 M-magnesium acetate. Another portion was adjusted to pH 6·7 and

similarly incubated with 0·1 ml. of the spleen diesterase in the presence of 0·01 M-sodium tartrate, which was added to suppress the monoesterase activity. The reaction mixtures were deproteinized by shaking with $CHCl_3$, concentrated and chromatographed on Whatman no. 1 filter paper with an ascending solvent mixture of 2 N-formic acid–6 M-NH_4Cl–propan-2-ol (15:15:70, by vol.). Since the preparations of diesterases contained some monoesterase activity, both nucleosides were produced in each incubation mixture. However, a spot with the absorption spectrum and phosphorus content of thymidylic acid was found only in the hydrolysates of the first and third peaks by the snake enzyme and of the first and second peaks by the spleen enzyme. The isomers had therefore been eluted from the column in the order pCpTpCp, pTpCpCp and pCpCpTp.

The solvent used for paper chromatography is suitable for use with mixtures containing appreciable amounts of salt. The solvent runs in two phases. The faster one is free of chloride and contains the thymidine spot. Deoxycytidine is concentrated at the interphase. Thymidylic acid and deoxycytidylic acid move as separate spots in the slower phase, with the thymidylic acid in front.

Group 8. This appeared to be a complex mixture and only one peak, T_2p_3 (2·5–3·5 l.), had a constant ratio between the absorptions at 280 and 260 mμ. The T_2p_3 obtained (1·6 moles/100 g.atoms of P) was probably only a fraction of the T_2p_3 in the original mixture.

Two-dimensional chromatography on paper

To separate the complex mixture of products we have investigated less laborious alternatives to column chromatography and have been able to obtain good separations by two-dimensional paper chromatography after removing the terminal-phosphate groups (cf. Smith & Markham, 1952). By these methods it is possible to study the frequencies of a variety of sequences with only about 20 mg. of DNA. The degradation was performed with 88 mg. of DNA, but the same number of chromatograms could have been run with less than 30 mg. DNA was dissolved in water (4 mg./ml.), degraded with diphenylamine and extracted with ether as described above. Most of the purines were removed by passing the solution through a 4 cm. × 1 cm. diam. column of Dowex-50 (H^+ form). The column was washed with 100–200 ml. of water until the extinction coefficient of the washings was less than 0·05. The combined eluate and washings were adjusted to pH 5·6 by adding N-NaOH, concentrated in a rotary evaporator (bath temp. 40°) and transferred to a suitable tube in a final volume of 6 ml. Then, 0·15 ml. of 0·2 M-EDTA, adjusted to pH 5·6 with NaOH, was added to act as a buffer and also as an activator of the phosphatase. The solution was incubated at 37° with about 200 units of prostatic phosphomonoesterase until it was certain that the release of inorganic phosphate had stopped (60 hr.). The final amount of inorganic phosphate was 73 % of the total P. The solution was shaken vigorously with a little $CHCl_3$ and the denatured protein was centrifuged and washed with 1–2 ml. of water. The washings were added to the supernatant and the solution was concentrated in a current of air at 37° to 1·5 ml.

An amount containing about 7 μmoles of total phosphate was applied to each sheet of acid-washed paper and chromatographed first with propan-2-ol–water (70:30, v/v), which was allowed to drip off the serrated edge for about 3 hr. The papers were dried at room temperature and again developed with the same solvent in the same direction until the solvent just reached the serrated edge. The papers were dried and 2-methylpropan-2-ol–conc. HCl–water (11:1:3, by vol.) was run at right angles until the solvent front almost reached the edge of the paper. The chromatogram was then dried by standing for at least 18 hr. at room temperature. Contact prints were made on Ilford 50M document paper by the light of a Chromatalamp (Hanovia Ltd., Slough, Bucks.), with the shortest exposure needed to obtain satisfactory prints. A tracing of such a print is shown in Fig. 4.

The absorbing areas of the chromatograms were cut out and weighed to compare their areas. They were eluted by soaking in 4 ml. of 0·03 M-HCl for 30 min. The extinction coefficients were then measured at 260, 267 and 280 mμ. Three or four

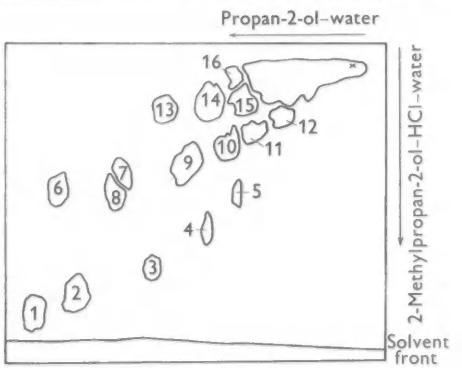

Fig. 4. Two-dimensional paper chromatogram of the products after degradation with diphenylamine and treatment wtih phosphomonoesterase. Solvents: propan-2-ol–water (70/30, v/v) and 2-methylpropan-2-ol–conc. HCl–water (11:1:3, by vol.). Tracing of photograph made in urltaviolet light. See Table 6 for the identities of the components.

blank areas of each chromatogram were similarly eluted as paper blanks. Except for the areas very close to the solvent front in the second solvent, the absorptions of these blanks were very similar per unit area of paper and so satisfactory blank corrections could be applied to the nucleotide eluates. The eluates from one of the chromatograms were used for determining total and inorganic phosphate. Most of the paper blank was due to inorganic phosphate. The results are summarized in Table 6.

The identities of the thymidine and deoxycytidine spots (numbers 1 and 6 in Fig. 4) were confirmed by paper-chromatographic comparisons and by their ultraviolet-absorption spectra at pH 1·5, 7·0 and 13·0. Thymidine and its nucleotides have a value of about 0·7 for the ratio of extinction at 280 mμ to that at 260 mμ at pH 1·5 whereas deoxycytidine and its nucleotides have values of about 2. The proportions of thymine to cytosine in the various components were obtained by comparing their ratio of extinctions at 280 and 260 mμ with the values calculated for various mixtures of integral molar proportions of thymidine and deoxycytidine. The total pyrimidine content of each component was obtained from the extinction at 267 mμ as described below. The ratio of phosphorus to pyrimidine served to decide whether a component was a di- or tri-nucleotide or a higher oligonucleotide. Chain lengths were assigned to the higher oligonucleotides on the basis of their chromatographic positions. Thus the row of spots 1–5 appears to be the homologous series of thymidine and the thymidine nucleotides of chain lengths 2–5. Spots 6, 13 and 16 are the homologous series of cytidine and the di- and tri-cytidine nucleotides. Spots 7 and 8 are the two isomers CpT and TpC, and the row of spots 7 plus 8 and 9–12 appear to be the homologous series of nucleotides containing only one cytidine and increasing amounts of thymidine. Similarly, the row from 13 to 15 appears to be the series with two cytidine residues and 0, 1 or 2 thymidine residues.

Spots 7 and 8 were clearly separated and, on hydrolysis with spleen diesterase and paper chromatography as described above, spot 7 gave a material with the ultraviolet-absorption and phosphate content of cytidylic acid (Cp). No thymidylic acid (Tp) was detected. Spot 8 gave thymidylic acid but no cytidylic acid. The two materials were therefore the two isomers CpT and TpC respectively.

The total amount of material applied to the chromatograms was obtained either by measurement of the volume of solution first applied or, more conveniently, by eluting the inorganic phosphate and determining it colorimetrically. The inorganic phosphate ran in the neighbourhood of spots 2 and 3 in the first solvent and in front of them in the second solvent. The two methods agreed very closely. For three chromatograms, the amounts of organic P for the identified spots were calculated from the ultraviolet absorption as described below. The organic P in the unidentified ultraviolet-absorbing areas was determined directly. The respective total recoveries were 74, 83 and 90 % of the organic P applied to the paper.

For comparison, Table 6 includes the amounts of the various nucleotides that are expected if the nucleotides are randomly arranged in the DNA. These random values were calculated according to Jones, Stacey & Watson (1957), taking 21·4 moles

Table 6. *Dephosphorylated pyrimidine nucleotide products separated by two-dimensional paper chromatography*

Spot no.	Identity	E_{280}/E_{260} Found	E_{280}/E_{260} Theory for mixture of T and C	Organic P/pyrimidine Found	Organic P/pyrimidine Theory	Amount (moles of pyrimidine/100 g.atoms of DNA P)	Mean	Theory for random
1	T	0·70	0·70	0	0	6·17, 6·3, 6·15, 6·2	6·2	7·07
2	T_2p	0·68	0·70	0·52	0·5	2·6, 2·65, 2·6, 2·6	2·6	4·00
3	T_3p_2	0·67	0·70	0·65	0·67	1·34, 1·48, 1·2, 1·21	1·3	1·70
4	T_4p_3	0·70	0·70	0·81	0·75	0·6, 0·57	0·6	0·64
5	T_5p_4	0·70	0·70	—	0·8	0·27	(0·3)	0·23
6	C	2·2	2·0	0	0	3·82, 3·84, 3·82, 3·75	3·8	5·35
7	CpT	1·25	1·24	0·54	0·5	5·03, 5·2, 5·01 {3·06	3·1	3·02
8	TpC	1·25	1·24	0·51	0·5	5·03, 5·2, 5·01 {1·95	2·0	3·02
9	CT_2p_2	1·03	1·04	0·67	0·67	2·7	2·7	3·85
10	CT_3p_3	0·95	0·95	0·77	0·75	1·6	1·6	1·94
11	CT_4p_4	0·85	0·89	0·7	0·8	0·75	0·7	0·86
12	CT_5p_5	0·83	0·86	0·88	0·83	0·49	0·5	0·35
13	C_2p	2·06	2·0	0·52	0·5	1·78, 1·80, 1·75	1·8	2·29
14	C_2Tp_2	1·5	1·46	0·65	0·67	2·6	2·6	2·92
15	$C_2T_2p_3$	1·25	1·24	0·78	0·75	1·9	1·9	2·20
16	C_2p_2	1·89	2·0	0·67	0·67	0·7	0·7	0·76

of cytosine and 28·3 moles of thymidine per 100 g.-atoms of DNAP. Differences of less than 0·3-mole/100 g.atoms of P are not regarded as being significant.

Evaluation of the amounts of nucleotides

Because the measurement of ultraviolet absorption is more reproducible and easier than is the determination of total phosphorus, the amounts of the various nucleotides in Tables 5 and 6 have been determined from their ultraviolet absorption. There are, however, some conflicting values for the molar-extinction coefficients. We have measured the millimolar-extinction coefficients of thymidine 5′-phosphate and 3′:5′-diphosphate at pH 1·0–2·0, obtaining their concentrations from the phosphorus content. For the materials supplied by the California Corporation for Biochemical Research (Los Angeles 63, Calif., U.S.A.) our values are 9·3 and 9·0 respectively at 260 mμ and 10·2 and 9·8 at 267 mμ. For the samples of thymidine diphosphate isolated in this work (Table 4) we obtained values of 9·3 and 9·9 at 260 mμ. The values for thymidylic acid are at least 10 % higher than those of Volkin & Cohn (1954) but they agree closely with those of Sinsheimer (1954) and of Shapiro & Chargaff (1957*a*). The values for thymidine 3′:5′-diphosphate at 260 mμ are appreciably higher than those of 8·64 and 8·2 previously reported by Shapiro & Chargaff (1957*a*) and by Cohn & Volkin (1957) respectively.

We have also examined the effect of enzymic dephosphorylation on the ultraviolet absorption of these two nucleotides. At 267 mμ, any change was less than 1 % at either pH 1·5 or 5·6 and so their extinction coefficients are, in fact, virtually the same and also very close to that of thymidine itself. Likewise, when deoxycytidine 5′-phosphate was dephosphorylated the change was less than 1 % at pH 1·5.

The amounts of the nucleotides of thymine or cytosine have accordingly been evaluated by using a value of 9·9 for the extinction coefficient per m-mole of total pyrimidine/l. at 267 mμ and pH 1·0–2·0. This value is reasonably consistent with the above-mentioned values for the two thymidine compounds and also with data for thymidine, cytidine and deoxycytidine (Fox & Shugar, 1952), for deoxycytidine 5′-phosphate and for its 3′:5′-diphosphate (Shapiro & Chargaff, 1957*a*) as well as for the 2′- and 3′-phosphates of cytidine (Loring, Bortner, Levy & Hammell, 1952). The same extinction coefficient has been used for the various oligonucleotides of cytosine and thymine. Although the absorption by oligonucleotides is usually less than that by a mixture of the component mononucleotides, it appears that this hypochromic effect is only about 3 % for nucleotides containing only thymine or cytosine or both (Privat de Garilhe & Laskowski, 1956; Gilham & Khorana, 1958). The values in Tables 5 and 6 support the view that this hypochromic effect can be neglected for the present purposes. For 5-methylcytidine 3′:5′-diphosphate the coefficient of 3·4 at 260 mμ and pH 1–2 has been used. This was taken to be the same as that for the 5′-phosphate and was obtained from the data of Sinsheimer (1954) and of Volkin & Cohn (1954). The material isolated had a value of 2·64 instead of 3·15 for the ratio of extinction at 280 mμ to that at 260 mμ. As mentioned above, base analyses indicated that it was mixed with about 18 % of the analogous cytosine compound.

Possibility of exchange reactions

Although it seems unlikely that the proportions of the different products can be appreciably affected by any exchange reactions occurring during the degradation or the enzymic dephosphorylation, such a possibility cannot be discounted without direct evidence. The following experiments with ^{32}P-labelled nucleotides indicate that the products do not undergo any appreciable degree of exchange by reactions that involve their phosphodiester groups.

Dr L. I. Fessler kindly gave us the insoluble fractions from *Escherichia coli* that had been grown on 1·25 l. of a synthetic growth medium containing $Na_2H^{32}PO_4$ (1 mc = 0·1 m-mole/l.) as the sole source of P and crushed in a bacterial press (Hughes, 1951). These fractions were suspended in water, shaken vigorously with octanol and $CHCl_3$ and centrifuged. The supernatant was poured into ethanol, the fibrous precipitate was washed with 80 % ethanol, suspended in water and the insoluble material was removed by centrifuging. Colorimetric estimation (Burton, 1956) indicated the presence of 5·8 μmoles of DNA P in the solution. It was treated with 0·4 N-NaOH for 6 hr. at 38° to degrade the ribonucleic acid. Calf-thymus DNA (10 mg.) was added as carrier and the DNA was precipitated by neutralization with HCl, addition of perchloric acid to a final concentration of 0·1 N and the addition of 0·3 vol. of ethanol. This ^{32}P-labelled DNA was washed with a mixture of 20 ml. of ethanol and 4 ml. of 2·5 N-perchloric acid at 0°. It was then degraded with diphenylamine and formic acid, extracted with ether and chromatographed on Dowex-1 essentially as for Fig. 2 but with a smaller column and smaller volumes. The four principal groups of nucleotides were: 1, pCp; 2, mainly C_2p_3; 3, mainly Tp_2; 4, a complex mixture similar to group 6 of Fig. 2 and Table 4. The fractions were concentrated and freed of salt by adsorption on charcoal as described in the Materials and Methods section.

A portion of each ^{32}P-labelled fraction was added to about 4 mg. of DNA and the mixture was treated with diphenylamine and dephosphorylated with phosphatase essentially as described above, but omitting to remove the purines by Dowex-50. Two-dimensional chromatograms were run and the radioactive areas detected by X-ray film. These patterns were compared with those on chromatograms obtained by first dephosphorylating the ^{32}P-labelled fraction and then adding to it some DNA that had already been treated with diphenylamine and phosphatase. Each fraction gave a similar pattern of radioactivity after both treatments.

The clearest results were given by the Cp_2 fraction, because this did not contain any other component. It was completely dephosphorylated by the enzyme and virtually all the radioactivity was found in the inorganic phosphate area even when the Cp_2 had been mixed with the DNA for treatment with diphenylamine and then the phosphatase. Thus during these reactions none of its phosphorus had been converted into phosphodiester groups and so neither of the following two reactions had taken place:

$$\text{-Py-p} + {}^{32}\text{p-C-}{}^{32}\text{p} \rightarrow \text{-Py-}{}^{32}\text{p-C-}{}^{32}\text{p} + \text{p}$$

$$ {}^{32}\text{p-C-}{}^{32}\text{p} + \text{p-Py-} \rightarrow {}^{32}\text{p-C-}{}^{32}\text{p-Py-} + \text{p}$$

Although the following reaction is not directly excluded,

$$\text{-Py-p} + {}^{32}\text{p-C-}{}^{32}\text{p} \rightarrow \text{-Py-p-C-}{}^{32}\text{p} + {}^{32}\text{p}$$

its occurrence is unlikely since it should have been accompanied by the second of the two excluded reactions. The absence of radioactivity in the diester groups is thus an indication that none of these exchange reactions has occurred. This conclusion is, of course, supported by the apparent similarities between the two radioautographs obtained with each of the other three fractions.

DISCUSSION

As would be expected, the first stage in the degradation of DNA is the removal of the purines. This is shown by the fact that the release of phosphate from apurinic acid occurs much sooner than from intact DNA. The mechanism of the subsequent release of phosphate remains obscure. Acid conditions are necessary. The reactions presumably involve the condensation of the amine with the aldehyde groups of the apurinic acid and it is possible to visualize a series of electron shifts along the deoxyribose chain resulting in the elimination of the phosphoester groups at first from the 3 position and then from the 5 position. The immediate product does not, however, seem to be stable and it undergoes further reactions. Under the conditions of the colorimetric reaction (Burton, 1956) it gives an intense blue colour but this does not happen in the formic acid mixture, where the colour is much fainter and is not intensified when the products are transferred to the conditions of the colorimetric reaction.

The evidence presented in this paper shows that the degradation of DNA by diphenylamine effectively results in the quantitative removal of the purine nucleoside components yielding inorganic orthophosphate, pyrimidine nucleoside 3′:5′-diphosphates and pyrimidine di- and oligonucleotide fragments with phosphomonoester groups at each end (Py_np_{n+1}). Although other reactions such as acid hydrolysis (Cohn & Volkin, 1957; Shapiro & Chargaff, 1957*a*, *b*) or mercaptalation and alkaline hydrolysis (Kent, Lucy & Ward, 1955; Jones & Letham, 1956; Jones, Letham & Stacey, 1956*a*, *b*) also give similar or analogous pyrimidine mono-, di- and oligo-nucleotide fragments, the diphenylamine reaction appears to go to completion without the occurrence of either appreciable side reactions or exchange processes. The diphenylamine reaction is therefore of much more value and it is very suitable for studying the patterns of nucleotide sequences in DNA.

The amounts of inorganic phosphate released from calf-thymus DNA, both with and without subsequent treatment with phosphatase, indicate a slight bias in favour of interpurine (-Pu-p-Pu-) and interpyrimidine (-Py-p-Py-) sequences, for they occur to the extent of 27–28% of the DNA phosphorus instead of the 25% expected on a random arrangement. By difference, the -Py-p-Pu- or -Pu-p-Py- sequences therefore represent 22–23% of the total DNA phosphorus.

The amounts of the nucleotides obtained by ion-exchange chromatography (Table 5) agree closely with those obtained by the paper-chromatographic method (Table 6). The results also demonstrate a bias in favour of polypyrimidine sequences because the amount of pyrimidines in the mono- and di-nucleotide fragment is 39% of the total, leaving 61% to be accounted for as trinucleotides and higher oligonucleotides. On a random arrangement, 50% would occur in each group. Perhaps the clearest evidence that the arrangement is not random is the considerable difference between the amounts of the two sequential isomers, pCpTp and pTpCp.

The amounts of the mono- and di-pyrimidine fragments recovered in this work are much higher than those obtained by Shapiro & Chargaff (1957*b*) after hydrolysing DNA in 0·1 M-sulphuric acid at 100° for 30 min.

The runs of four and five thymine nucleotides with a purine nucleotide at each end are found in amounts which are somewhat similar to those which would occur in a randomly arranged poly-

nucleotide. These findings have a bearing on the feasibility of certain codes which have been suggested as conceivable relations between the sequences of the nucleotides in DNA and those of the amino acids in proteins (Crick, Griffith & Orgel, 1957; Golomb, Welch & Delbrück, 1958).

SUMMARY

1. A number of aromatic amines have been found to induce the release of inorganic orthophosphate from deoxyribonucleic acid in aqueous 66% (v/v) formic acid at 30°. The reaction with diphenylamine, the most effective of the amines tested, has been studied in detail.

2. The purines are first removed and then both phosphoester bonds attached to the exposed deoxyribose residues are broken. Phosphodiester groups joining two purine nucleoside residues are converted into inorganic phosphate. Sequences of pyrimidine nucleotides appear quantitatively as nucleoside 3′:5′-diphosphates and as di- and oligonucleotides with terminal phosphate groups at both ends.

3. After enzymic removal of the terminal phosphate groups, many of the pyrimidine oligonucleotides can be separated by two-dimensional chromatography on paper, thus providing a simple method for the study of the frequencies of certain sequences of nucleotides.

4. In calf-thymus deoxyribonucleic acid there is a slight bias in favour of three or more consecutive pyrimidine nucleotides. These amount to about 61% of the total pyrimidines instead of the 50% for a random arrangement.

5. Evidence is given that runs of four and five thymine nucleotides with a purine nucleotide at each end occur in amounts which are somewhat similar to those of a random arrangement.

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REFERENCES

Bartley, W. (1953). *Biochem. J.* **54**, 677.
Berenblum, I. & Chain, E. (1938). *Biochem. J.* **32**, 295.
Boman, H. G. (1955). *Biochim. biophys. Acta*, **16**, 245.
Burton, K. (1956). *Biochem. J.* **62**, 315.
Burton, K. & Petersen, G. B. (1957). *Biochim. biophys. Acta*, **26**, 667.
Chargaff, E. (1955). In *The Nucleic Acids*, vol. 1, p. 307. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press Inc.
Chen, P. S., Toribara, J. Y. & Warner, H. (1956). *Analyt. Chem.* **28**, 1756.
Cohn, W. E. & Volkin, E. (1957). *Biochim. biophys. Acta*, **24**, 359.
Cohn, W. E., Volkin, E. & Khym, J. X. (1957). *Biochem. Prep.* **5**, 49.
Crick, F. H. C., Griffith, J. S. & Orgel, L. E. (1957). *Proc. nat. Acad. Sci., Wash.*, **43**, 416.
Davidson, H. M. & Fishman, W. H. (1959). *J. biol. Chem.* **234**, 526.
Fox, J. J. & Shugar, D. (1952). *Biochim. biophys. Acta*, **9**, 369.
Gilham, P. T. & Khorana, H. G. (1958). *J. Amer. chem. Soc.* **80**, 6212.
Golomb, S. W., Welch, L. R. & Delbrück, M. (1958). *Biol. Medd., Kbh.*, **23**, 1.
Heppel, L. A. & Hilmoe, R. J. (1955). In *Methods in Enzymology*, vol. 2, p. 565. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Hughes, D. E. (1951). *Brit. J. exp. Path.* **32**, 97.
Hughes, T. R. & Klotz, I. M. (1956). *Meth. biochem. Anal.* **3**, 265.
Jones, A. S. & Letham, D. S. (1956). *J. chem. Soc.* p. 2573.
Jones, A. S., Letham, D. S. & Stacey, M. (1956*a*). *J. chem. Soc.* p. 2579.
Jones, A. S., Letham, D. S. & Stacey, M. (1956*b*). *J. chem. Soc.* p. 2584.
Jones, A. S., Stacey, M. & Watson, B. E. (1957). *J. chem. Soc.* p. 2454.
Kent, P. W., Lucy, J. A. & Ward, P. F. V. (1955). *Biochem. J.* **61**, 529.
Kerr, S. W. & Chernigoy, F. (1957). *J. biol. Chem.* **228**, 495.
Loring, H. S., Bortner, H. W., Levy, L. W. & Hammell, M. L. (1952). *J. biol. Chem.* **196**, 807.
Markham, R. (1955). In *Moderne Methoden der Pflanzenanalyse*, vol. 4, p. 246. Ed. by Paech, K. & Tracey, M. W. Berlin: Springer-Verlag.
Moore, S. & Stein, W. H. (1951). *J. biol. Chem.* **192**, 663.
Parr, C. W. (1954). *Biochem. J.* **56**, xxvii.
Privat de Garilhe, M., Cunningham, L., Laurila, U.-R. & Laskowski, M. (1957). *J. biol. Chem.* **224**, 751.
Privat de Garilhe, M. & Laskowski, M. (1955). *Biochim. biophys. Acta*, **18**, 370.
Privat de Garilhe, M. & Laskowski, M. (1956). *J. biol. Chem.* **223**, 661.
Shapiro, H. S. & Chargaff, E. (1957*a*). *Biochim. biophys. Acta*, **26**, 596.
Shapiro, H. S. & Chargaff, E. (1957*b*). *Biochim. biophys. Acta*, **26**, 608.
Sinsheimer, R. L. (1954). *J. biol. Chem.* **208**, 445.
Smith, J. D. & Markham, R. (1952). *Biochim. biophys. Acta*, **8**, 350.
Tamm, C., Hodes, M. E. & Chargaff, E. (1952). *J. biol. Chem.* **195**, 49.
Volkin, E. & Cohn, W. E. (1954). *Meth. biochem. Anal.* **1**, 287.
Wyatt, G. R. (1955). In *The Nucleic Acids*, vol. 1, p. 243. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press Inc.

Biochem. J. (1960) **75**, 28

Biochemical Studies of Toxic Agents

12. THE BIOSYNTHESIS OF *n*-PROPYLMERCAPTURIC ACID FROM *n*-PROPYL HALIDES*

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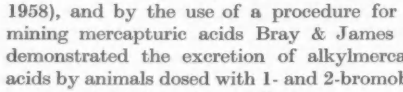
Before the preliminary account of the present work (Grenby & Young, 1959) there appears to have been no report of the isolation of a biosynthetic alkylmercapturic acid. Evidence had been obtained, however, which suggested that alkylmercapturic acids could probably be isolated from the urine of animals which had been dosed with certain alkylating agents. Roberts & Warwick (1957, 1958), by means of radiochromatography, showed the presence of ethylmercapturic acid in the urine of rats which had been dosed with ethyl methanesulphonate. By chromatographic and radiochromatographic methods ethylmercapturic acid was also detected in the urine of rats after administration of bromoethane (Thomson, Maw & Young, 1958), and by the use of a procedure for determining mercapturic acids Bray & James (1958) demonstrated the excretion of alkylmercapturic acids by animals dosed with 1- and 2-bromobutane and certain higher 1-bromoalkanes.

The present work was undertaken to determine whether the three *n*-propyl halides, 1-chloro-, 1-bromo- and 1-iodo-propane, give rise in the animal body to *n*-propylmercapturic acid, i.e. *N*-acetyl-*S*-(*n*-propyl)-L-cysteine. 1-Fluoropropane (b.p. $-3{\cdot}2°$) was not studied.



(X = Cl, Br or I)

By means of paper chromatography *n*-propylmercapturic acid was detected in the urine of rats, rabbits, guinea pigs and mice dosed with 1-bromo- or 1-iodo-propane, and in the urine of rats and rabbits dosed with 1-chloropropane. The mercapturic acid was isolated from the urine of rats which had been dosed with 1-bromo- or 1-iodopropane.

EXPERIMENTAL AND RESULTS

All melting points are uncorrected.

Preparation of S-(n-*propyl*)-L-*cysteine*

Method I. The procedure was based on that described by Armstrong & Lewis (1951) and depended on the reduction of L-cystine by sodium in anhydrous ammonia (du Vigneaud, Audrieth & Loring, 1930) followed by *S*-propylation of the cysteine by means of 1-iodopropane. Small pieces of metallic sodium were added, with stirring, to 2 g. of L-cystine dissolved in 100 ml. of anhydrous ammonia cooled in a mixture of solid CO_2 and ethanol, until a permanent deep-blue colour was produced. With continued stirring 0·2–0·4 ml. portions of 1-iodopropane were added at intervals of about 5 min. until a sample of the reaction mixture gave a negative nitroprusside test for the presence of thiol groups, 2·2 ml. of 1-iodopropane being required to achieve this. The ammonia was allowed to evaporate at room temperature, leaving a white residue which was then dissolved in the minimum volume of water. The pH of the solution was adjusted to 5·0 by the addition of conc. HCl and the precipitate which formed was separated and crystallized first from 85% (v/v) methanol in water and then from water. The product consisted of colourless glistening plates of *S*-(*n*-propyl)-L-cysteine in an amount corresponding to 65% of the theoretical yield; m.p. 241–243° (decomp.); $[\alpha]_D^{20} - 22°$ in water (*c*, 2); $[\alpha]_D^{20} - 5°$ in N-HCl (*c*, 1) (Found: C, 44·4; H, 8·1; N, 8·6; S, 19·3. Calc. for $C_6H_{13}O_2NS$: C, 44·1; H, 8·0; N, 8·6; S, 19·6%). The compound was soluble in water to the extent of 2·5 g./100 ml. at 22°, but was only very sparingly soluble in the common organic solvents.

Method II. This method was similar to that of Stoll & Seebeck (1948) and Roberts & Warwick (1958), and involved the interaction of 1-iodopropane and L-cysteine in an aqueous ethanol solution of NaOH. The yield of *S*-(*n*-propyl)-L-cysteine was 21%; m.p. 245–247° (decomp.); $[\alpha]_D^{20} - 22°$ in water (*c*, 2) (Found: C, 44·3; H, 7·9; N, 8·6; S, 19·8%).

Preparation of n-*propylmercapturic acid*

Method I. To 3 g. of *S*-(*n*-propyl)-L-cysteine in 9 ml. of water was added 4·5 ml. of 5N-NaOH, and the solution was cooled to just below 5°. The temperature was maintained at this level while a steady stream of keten was bubbled through the solution. When necessary, drops of 5N-NaOH were added to keep the solution just alkaline to phenolphthalein. After keten had passed through the solution for 30 min. the solution no longer gave a positive ninhydrin test. It was then made to pH 2·0 by the addition of conc. HCl and extracted by shaking three times with three-volume portions of chloroform. The chloroform extracts were combined and, when evaporated to dryness, yielded

* Part 11: Knight & Young (1958).

a gummy residue which gradually solidified. This was crystallized twice from a small volume of water and twice from benzene; the yield was 65% of the theoretical amount of n-*propylmercapturic acid*. The product was in the form of needles, m.p. 96°; $[\alpha]_D^{22} - 19°$ in water (*c*, 2) (Found: C, 46·6; H, 7·2; N, 6·7; S, 15·9. $C_8H_{15}O_3NS$ requires C, 46·8; H, 7·4; N, 6·8; S, 15·6%).

Method II. The procedure resembled that described by du Vigneaud, Wood & Binkley (1941) for the acetylation of *p*-bromophenyl-L-cysteine by acetic anhydride in the presence of NaOH. Starting from 0·267 g. of *S*-(*n*-propyl)-L-cysteine the yield of mercapturic acid was 57%; m.p. 95°; $[\alpha]_D^{23} - 18°$ in water (*c*, 2) (Found: C, 46·7; H, 7·3; N, 7·1; S, 15·9%).

Method III. *N*-Acetyl-L-cysteine, m.p. 110°, was prepared by a method based on that of Pirie & Hele (1933), in which L-cystine is acetylated by keten and is then reduced to *N*-acetyl-L-cysteine. *n*-Propylmercapturic acid was prepared by allowing 3 ml. of 1-iodopropane to react with 2·2 g. of *N*-acetyl-L-cysteine in anhydrous ammonia under conditions similar to those already described under method I for the preparation of *S*-(*n*-propyl)-L-cysteine. The yield was 72%; m.p. 96°; $[\alpha]_D^{20} - 19°$ in water (*c*, 2) (Found: C, 47·2; H, 7·1; N, 6·9; S, 15·4%).

n-Propylmercapturic acid had the following solubilities at 20° (in g./100 ml. of solution): water, 5·6; ethanol, 28·3; ether, 2·7; chloroform, 24·0; benzene, < 0·1.

Deacetylation of n-*propylmercapturic acid*

A step which was used in the identification of *n*-propylmercapturic acid in the present work was its conversion into *S*-(*n*-propyl)-L-cysteine. This deacetylation was brought about by acid hydrolysis and also enzymically.

By acid hydrolysis. To 0·151 g. of synthetic *n*-propylmercapturic acid were added 2 ml. of water and 8 ml. of conc. HCl. The solution was heated in a boiling-water bath for 5 hr. and was then concentrated until crystallization began. The hydrolysate was made to pH 5·0 at room temperature by the addition of NaOH solution, concentrated again and left in the refrigerator. The plates which separated were recrystallized from a small volume of water, filtered and washed with water and with acetone. The yield of *S*-(*n*-propyl)-L-cysteine was 33% of the theoretical; m.p. 239–241° (decomp.); the mixed m.p. with the authentic compound was 239–240° (decomp.); $[\alpha]_D^{24} - 22°$ in water (*c*, 2) (Found: C, 44·5; H, 8·1; N, 8·7; S, 19·8%).

By enzymic hydrolysis. A variety of acetamido compounds have been shown to undergo deacetylation when incubated with tissue extracts (see Bray, James, Thorpe & Wasdell, 1950). The enzymic deacetylation of *N*-acetyl-*S*-benzyl-L-cysteine was demonstrated by Birnbaum & Greenstein (1952). In the present work it was found that when *n*-propylmercapturic acid was incubated with an extract of the liver or kidney of the rat it was deacetylated. The preparation of the tissue extract and the conditions of hydrolysis were similar to those described by Bray, James, Ryman & Thorpe (1948) for the enzymic hydrolysis of amides.

The liver (7·3 g.) of a freshly killed rat was ground with a little fine sand and about twice its weight of water, and centrifuged at 1000 *g* for 15 min. The supernatant liquid was decanted and added to a solution of 0·496 g. of *n*-propylmercapturic acid in 50 ml. of water, 8 ml. of 0·2N-Na_2HPO_4 and 2 ml. of 0·2N-NaH_2PO_4. The mixture was adjusted to pH 7·4 and incubated at 37° for 24 hr. It was boiled and filtered, and the filtrate was shaken first with an equal volume of chloroform, then with an equal volume of ether. The aqueous solution was evaporated until crystals appeared and was left in the refrigerator. The precipitate was recrystallized twice from water, filtered and washed with acetone. The product consisted of plates in a yield corresponding to 12% of the theoretical, m.p. 242–243° (decomp.) (Found: C, 44·3; H, 7·5; N, 8·5; S, 20·0%). No such product was obtained in control experiments in which either the tissue extract or *n*-propylmercapturic acid was omitted. The conditions for deacetylation of the mercapturic acid by extracts of rat kidney were similar to those described for liver.

Cleavage of n-*propylmercapturic acid by alkali*

Although fairly resistant to acid hydrolysis, *n*-propylmercapturic acid is readily broken down by NaOH, with formation of the sodium salt of *n*-propanethiol. This thiol can be characterized as *n*-propanethiol mercury chloride, $CH_3{\cdot}CH_2{\cdot}CH_2{\cdot}S{\cdot}HgCl$ (Challenger & Rawlings, 1937), which was prepared by mixing 0·42 ml. of *n*-propanethiol with 12 ml. of 15% (w/v) mercuric chloride in methanol. The mixture was kept for 24 hr. with occasional shaking and the white precipitate which formed was filtered and crystallized from a large volume of ethanol. Small sparkling plates were obtained in a yield corresponding to 43% of the theoretical, m.p. 181° (decomp.). Challenger & Rawlings (1937) reported that the compound melted at 182° (decomp.) (Found: C, 11·4; H, 2·4; S, 10·4. Calc. for C_3H_7ClSHg: C, 11·6; H, 2·3; S, 10·3%).

A solution of 0·466 g. of *n*-propylmercapturic acid in 5 ml. of 15% (w/v) NaOH in water was heated in a water bath at 90° for 15 min. The yellow solution thus obtained was cooled in ice, and conc. HCl was added drop by drop until the solution became turbid and the odour of the thiol markedly increased. On the addition of 6 ml. of 15% (w/v) mercuric chloride in methanol a pale-yellow solid appeared. This was crystallized from ethanol and gave white crystals in a yield corresponding to 24% of the theoretical; m.p. 182° (decomp.), unchanged by admixture with authentic *n*-propanethiol mercury chloride prepared as already described (Found: C, 11·8; H, 2·2; S, 10·3%).

Animals and dosing

Rats, rabbits, guinea pigs and mice were housed in metabolism cages so constructed that the urine was collected separately from the faeces. Urine was collected for 24 hr. after dosing, or for 48 hr. from rabbits, and when not used immediately it was stored in the refrigerator. Rats, rabbits and mice were given a diet of rat cakes [J. Murray and Sons (London) Ltd.] and had access to water at all times. The rabbits received cabbage in addition. Guinea pigs were fed on a mixture of bran and oats and were also given cabbage.

1-Chloropropane, 1-bromopropane and 1-iodopropane (British Drug Houses Ltd.) were redistilled. They were administered to the animals, either undiluted or as a 40% (w/v) solution in arachis oil or liquid paraffin, by subcutaneous injection in the dorsal region. With the exception of the rabbits, the animals were anaesthetized lightly with ether whilst being dosed.

Chromatographic studies

In early experiments the urine of each group of animals dosed with an *n*-propyl halide was examined by paper chromatography for the presence of *n*-propylmercapturic acid. In later work concentrates of chloroform extracts of the acidified urine were used instead so as to reduce interference by other urinary constituents.

General methods. Urine extracts were prepared by acidifying the urine with HCl to pH 2·0 and shaking it three times with three-volume portions of chloroform. The combined chloroform extracts were evaporated to a small volume, and suitable amounts of the concentrate were applied to the paper for testing for the presence of the mercapturic acid. Some of the chloroform extract was evaporated to dryness and subjected to acid hydrolysis under the conditions described earlier, with appropriate reduction in volumes. The hydrolysate was examined by paper chromatography for the presence of *S*-(*n*-propyl)-L-cysteine. It was recognized, however, that whereas the absence of *S*-(*n*-propyl)-L-cysteine from the acid hydrolysate of the urine or urine extract was evidence against the presence of *n*-propylmercapturic acid in the original urine, *S*-(*n*-propyl)-L-cysteine might appear in the hydrolysate as a result of the breakdown of compounds other than the mercapturic acid. The chromatograms were developed on Whatman no. 1 paper by the ascending method for 16 hr. at room temperature. On each chromatogram synthetic *n*-propylmercapturic acid and *S*-(*n*-propyl)-L-cysteine were run as reference compounds.

Detecting reagents. Two reagents were used, both of which detect *n*-propylmercapturic acid and *S*-(*n*-propyl)-L-cysteine. One of these was the $K_2Cr_2O_7$–$AgNO_3$ reagent described by Knight & Young (1958) and the other was 0·1 N-$KMnO_4$. When the chromatogram was sprayed lightly with 0·1 N-$KMnO_4$ a pink background was produced with yellow areas marking the positions of the mercapturic acid and the *S*-(*n*-propyl)-L-cysteine. The colours faded within a few minutes, however, and in this respect this reagent was less satisfactory than the $K_2Cr_2O_7$–$AgNO_3$ reagent.

Solvent mixtures. (A) The upper layer obtained after shaking a mixture of equal parts of butan-1-ol (AnalaR) and 1·33 N-acetic acid. (B) Pyridine–water–aq. NH_3 soln. sp.gr. 0·88 (20:2:1, by vol.). The following R_F values (each the mean of not less than 30 measurements) were obtained: *n*-propylmercapturic acid, (A) 0·81; (B) 0·67; *S*-(*n*-propyl)-L-cysteine, (A) 0·41; (B) 0·37.

Results. Male rats, rabbits, guinea pigs and mice were injected subcutaneously with a 40% (w/v) solution of the 1-halogenopropane in arachis oil and their urine was examined by means of paper chromatography for the presence of *n*-propylmercapturic acid (see Table 1). Samples of the normal urine of each species studied, or of the urine excreted after the administration of the diluent alone, showed no evidence for the presence of *n*-propylmercapturic acid.

Isolation of n-*propylmercapturic acid from the urine of rats dosed with* 1-*bromopropane*

After administration of 1-*bromopropane dissolved in a diluent.* A 40% (w/v) solution of 1-bromopropane in liquid paraffin was injected subcutaneously into 18 rats (150–200 g. body wt.) so that each received 0·25 g. of the halogenopropane. The dosing was repeated on three further occasions at intervals of 48 hr. and the urine excreted in the 24 hr. immediately following each dosing was collected. The urine, total volume 520 ml., was filtered and made acid to Congo red by the addition of conc. HCl. The acidified urine was shaken on a mechanical shaker for 30 min. with 3 vol. of chloroform and the extract was separated after centrifuging to break the emulsion. The extraction was repeated twice more and the combined chloroform extracts were then evaporated to 50 ml. The dark-red solution thus obtained was shaken with 20 ml. of M-$NaHCO_3$, then with 10 ml. of M-$NaHCO_3$. The combined bicarbonate extracts were brought to pH 2·0 by the addition of conc. HCl, and the acidified solution was shaken three times with 3 vol. of chloroform. These chloroform extracts were evaporated to dryness and, after standing over P_2O_5, yielded 1·197 g. of dark-brown gum. When this was stirred with a few millilitres of ether a buff-coloured solid formed. This was separated by filtration and washed with a small volume of ether. When the solid (0·438 g.) was treated with 5 ml. of ethyl acetate part went into solution, leaving undissolved material which was separated by filtration and identified as hippuric acid. Light petroleum (b.p. 40–60°) was added

Table 1. *Excretion of* n-*propylmercapturic acid by animals dosed with* 1-*halogenopropanes*

All the animals were dosed with a 40% (w/v) solution of the 1-halogenopropane in arachis oil by subcutaneous injection in the dorsal region. The urine was examined by paper chromatography as described in the text. Detection of mercapturic acid: ++, readily detected; +, detected with difficulty; −, not detected.

Species	Body wt. (g.)	1-Halogeno-propane administered	Dose (g./kg. body wt.)	*n*-Propyl-mercapturic acid in urine
Rat	150–200	Chloro-	1·5	+
		Bromo-	1·5	++
		Iodo-	1·0	++
Rabbit	2400–3200	Chloro-	1·3	+
		Bromo-	1·0	++
		Iodo-	1·2	++
Guinea pig	300–450	Chloro-	1·8	−
		Bromo-	1·8	++
		Iodo-	1·3	++
Mouse	24–28	Chloro-	1·2	−
		Bromo-	1·2	++
		Iodo-	1·2	++

drop by drop to the ethyl acetate solution with stirring, and dark gummy material separated and adhered to the sides of the beaker. When enough light petroleum had been added to render the solution almost colourless, the clear solution was decanted and more light petroleum was added with stirring. This yielded 0·216 g. of an almost white solid, which was crystallized from benzene. The crystals were dissolved in ethanol and the solution was treated with charcoal and filtered. The filtrate was evaporated to dryness and the residue was crystallized from water. This yielded 0·081 g. of needles, m.p. 95°, unchanged by admixture with synthetic *n*-propylmercapturic acid; $[\alpha]_D^{20}$ – 19° in water (*c*, 2) (Found: C, 46·9; H, 7·0; N, 7·1; S, 15·8; equiv. 207. Calc. for $C_8H_{15}O_3NS$: C, 46·8; H, 7·4; N, 6·8; S, 15·6%; equiv. 205).

The R_F values obtained for the isolated compound in solvent mixtures (A) and (B) corresponded to those given by synthetic *n*-propylmercapturic acid run on the same chromatograms. After the isolated compound had been hydrolysed by heating with HCl, the hydrolysate contained a compound with the same R_F values in solvent mixtures (A) and (B) as those of *S*-(*n*-propyl)-L-cysteine. No *n*-propylmercapturic acid was obtained from the urine in a control experiment in which 36 rats were treated in the same way as in the experiment just described except that liquid paraffin alone was injected and only on two occasions.

After administration of 1-bromopropane without a diluent. In this experiment 66 rats (290–340 g. body wt.) were each injected subcutaneously on three occasions with 0·25 g. of 1-bromopropane alone, and the urine was collected for 24 hr. after each dosing. The urine was acidified and treated by a procedure which closely resembled that already described for the isolation of *n*-propylmercapturic acid, except that use was made of 1:2-dichloroethane as a solvent for crystallization. This yielded 0·488 g. of crystalline product, m.p. 94°; mixed m.p. with synthetic *n*-propylmercapturic acid 95°; $[\alpha]_D^{20}$ – 18° in water (*c*, 2). The R_F values of the product in solvent mixtures (A) and (B) corresponded to those of synthetic *n*-propylmercapturic acid run on the same chromatograms.

The acid hydrolysis of 0·208 g. of the compound isolated from urine yielded 0·073 g. of crystalline product corresponding to 44% of the theoretical amount of *S*-(*n*-propyl)-L-cysteine, m.p. 238–239° (decomp.), unchanged by admixture with synthetic *S*-(*n*-propyl)-L-cysteine; $[\alpha]_D^{23}$ – 22° in water (*c*, 2) (Found: C, 44·4; H, 7·7; N, 8·2; S, 19·4%). The R_F values of the hydrolysis product in solvent mixtures (A) and (B) were the same as those of synthetic *S*-(*n*-propyl)-L-cysteine run on the same chromatograms.

Alkaline hydrolysis of 0·197 g. of the compound isolated from urine gave a solution from which a mercury derivative was precipitated. When crystallized twice from ethanol this product gave 0·043 g. of small plates, m.p. 182° (decomp.); the mixed m.p. with synthetic *n*-propanethiol mercury chloride was 181° (decomp.) (Found: C, 11·9; H, 2·6; S, 10·3; Cl, 11·5. Calc. for C_3H_7ClSHg: Cl, 11·4%).

The properties of the compound isolated from urine, together with its behaviour on acid and alkaline hydrolysis, establish that it was *n*-propylmercapturic acid.

Isolation of n-*propylmercapturic acid from the urine of rats dosed with* 1-*iodopropane*

Thirty-three rats (230–260 g. body wt.) were each injected subcutaneously on two occasions with 0·25 g. of 1-iodopropane without a diluent, and the urine was collected for 24 hr. after each dosing. The urine was acidified and extracted with chloroform, and the extracts were fractionated as described for the isolation of *n*-propylmercapturic acid from the urine of rats dosed with 1-bromopropane. This yielded 0·058 g. of crystals, m.p. 95°, unchanged by admixture with synthetic *n*-propylmercapturic acid; $[\alpha]_D^{20}$ – 20° in water, (*c*, 2) (Found: C, 47·2; H, 7·7; N, 6·5; S, 15·6%). The R_F values of the compound in solvent mixtures (A) and (B) were the same as those of synthetic *n*-propylmercapturic acid run on the same chromatograms. When the compound was hydrolysed with HCl the hydrolysate gave chromatograms corresponding to those of *S*-(*n*-propyl)-L-cysteine.

In another experiment 33 rats (250–280 g. body wt.) were each injected subcutaneously on two occasions with 0·25 g. of 1-iodopropane. From the urine excreted in the 24 hr. periods after dosing, 0·229 g. of crystalline product was isolated by the method already described, m.p. 95°, unchanged by admixture with synthetic *n*-propylmercapturic acid; $[\alpha]_D^{23}$ – 19° in water (*c*, 1) (Found: C, 46·4; H, 7·4; N, 6·8; S, 15·9%). A portion (0·190 g.) of the isolated compound was deacetylated by incubation with a tissue extract as described earlier, except that rat kidney (1·7 g.) instead of liver was used. A crystalline product was separated weighing 0·050 g., m.p. 236–238° (decomp.); m.p. when mixed with synthetic *S*-(*n*-propyl)-L-cysteine 237–239° (decomp.); $[\alpha]_D^{20}$ – 21° in water, (*c*, 1) (Found: C, 44·2; H, 7·9; N, 8·7; S, 19·7%).

When the procedure for isolating the mercapturic acid was applied to 555 ml. of normal rat urine, this compound was not found.

Attempted isolation of n-*propylmercapturic acid from the urine of rats dosed with* 1-*chloropropane*

As the results obtained by chromatographic examination of the urine of rats dosed with 1-chloropropane indicated the presence of a low concentration of *n*-propylmercapturic acid, an attempt was made to isolate the mercapturic acid from the urine. Each rat of a group of 66 (190–240 g. body wt.) was injected subcutaneously on three occasions with 0·25 g. of 1-chloropropane, so that in all almost 50 g. of the compound were administered. The urine was collected for 24 hr. after each dosing, acidified and extracted with chloroform. The chloroform extracts gave evidence of the presence of *n*-propylmercapturic acid when examined by paper chromatography. When they were fractionated as already described, however, no product was obtained at the point in the process at which *n*-propylmercapturic acid was isolated from the urine of rats dosed with 1-bromo- or 1-iodo-propane.

DISCUSSION

The physical and chemical properties of the compound isolated from the urine of rats which had been dosed with 1-bromo- or 1-iodo-propane compared with those of the authentic mercapturic acid, clearly establish that the isolated compound was *n*-propylmercapturic acid. The compound was isolated from urine which had been acidified, however, and it is known that some mercapturic acids are not excreted as such, but are derived from

precursors which are broken down when the urine is acidified. These acid-labile precursors of mercapturic acids have been designated 'premercapturic acids' (Knight & Young, 1958) and that formed from naphthalene has been isolated and identified as *N*-acetyl-*S*-(1:2-dihydro-2-hydroxynaphthyl)-L-cysteine by Boyland & Sims (1958). All the compounds shown so far to give rise to premercapturic acids *in vivo* are aromatic, however, and for the aralkyl compound, benzyl chloride, there is evidence that it is excreted as a mercapturic acid and not as a premercapturic acid (Knight & Young, 1958). At present there are no grounds for believing that an alkyl halide is converted into an acid-labile compound from which the alkylmercapturic acid is liberated. This is supported by the observation that when the unacidified urine of animals which had been dosed with a 1-halogenopropane was examined by paper chromatography with a basic solvent system, pyridine–water–ammonia solution (sp.gr. 0·88) (20:2:1), a spot was obtained with R_F corresponding to that of *n*-propylmercapturic acid run on the same chromatogram. In the absence of evidence to the contrary, it would seem reasonable therefore to assume that *n*-propylmercapturic acid is a constituent of the urine of rats after the administration of 1-bromo- or 1-iodo-propane.

An interesting feature of work on the hydrolysis of *n*-propylmercapturic acid was the observation that this compound undergoes deacetylation when incubated with extracts of the liver or kidney of the rat. Evidence that deacetylation of a mercapturic acid can occur *in vivo* was obtained by Parke & Williams (1951), who reported the excretion of *S*-phenyl-L-cysteine by rabbits to which phenylmercapturic acid had been administered. The finding that enzymic deacetylation of *n*-propylmercapturic acid can occur raises the question of whether this has a bearing on the amount of the mercapturic acid excreted by animals dosed with a 1-halogenopropane. There is a possibility that this deacetylation process occurs only to a small extent in the intact animal, and that it is favoured by the conditions under which it is studied *in vitro*; evidence that such a situation can occur was obtained by Krebs, Sykes & Bartley (1947) in their study of the deacetylation of the *p*-acetamido group of sulphonamide drugs by animal tissues.

The amounts of *n*-propylmercapturic acid isolated from the urine represented the conversion of from 0·27 to 1·14% of the administered halogenopropane to the mercapturic acid. Even allowing for the fact that the isolation process was carried out with the object of obtaining an analytically pure product, it would seem that mercapturic acid formation accounts for the fate of no more than a small proportion of the administered halogenopropane. This may be attributable to other factors in addition to the influence of competing metabolic reactions. For example, it is probable that the volatility of the 1-halogenopropanes leads to some loss of the compounds through the lungs. This is of special significance with respect to 1-chloropropane, b.p. 46·6°, and could help to explain the low concentration of *n*-propylmercapturic acid in the urine of rats and rabbits dosed with 1-chloropropane, and the apparent absence of the mercapturic acid from the urine of guinea pigs and mice dosed with this compound. In view of this finding with guinea pigs it is of interest that Bray, Franklin & James (1959) have pointed out that mercapturic acid excretion by the guinea pig is much lower than that by the rat and rabbit under similar conditions.

Current interest in the biochemistry of alkylating agents stems mainly from the variety of biological effects which they produce and their significance in relation to the cancer problem. Their metabolism is being studied in the belief that the information gained may help to explain their action in the organism. The biosynthesis of *n*-propylmercapturic acid from a 1-halogenopropane provides a clear-cut example of the alkylation of the thiol group *in vivo*.

SUMMARY

1. *S*-(*n*-Propyl)-L-cysteine has been prepared by two methods, and n-*propylmercapturic acid*, i.e. *N*-acetyl-*S*-(*n*-propyl)-L-cysteine, by three methods.

2. The hydrolysis of *n*-propylmercapturic acid by acid and by alkali has been studied, and its conversion into *S*-(*n*-propyl)-L-cysteine by incubation with extracts of rat liver or kidney has been demonstrated.

3. By means of paper chromatography evidence has been obtained for the excretion of *n*-propylmercapturic acid by rats, rabbits, guinea pigs and mice dosed by subcutaneous injection with 1-bromopropane or 1-iodopropane, and by rats and rabbits, but not by guinea pigs and mice, injected subcutaneously with 1-chloropropane.

4. *n*-Propylmercapturic acid has been isolated from the urine excreted by rats in the 24 hr. after they had been injected subcutaneously with 1-bromopropane or 1-iodopropane.

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REFERENCES

Armstrong, M. D. & Lewis, J. D. (1951). *J. org. Chem.* **16**, 749.
Birnbaum, S. M. & Greenstein, J. P. (1952). *Arch. Biochem. Biophys.* **39**, 108.
Boyland, E. & Sims, P. (1958). *Biochem. J.* **68**, 440.
Bray, H. G., Franklin, T. J. & James, S. P. (1959). *Biochem. J.* **71**, 690.
Bray, H. G. & James, S. P. (1958). *Biochem. J.* **69**, 24P.
Bray, H. G., James, S. P., Ryman, B. E. & Thorpe, W. V. (1948). *Biochem. J.* **42**, 274.
Bray, H. G., James, S. P., Thorpe, W. V. & Wasdell, M. R. (1950). *Biochem. J.* **47**, 483.
Challenger, F. & Rawlings, A. A. (1937). *J. chem. Soc.* p. 868.
du Vigneaud, V., Audrieth, L. F. & Loring, H. S. (1930). *J. Amer. chem. Soc.* **52**, 4500.
du Vigneaud, V., Wood, J. L. & Binkley, F. (1941). *J. biol. Chem.* **138**, 369.
Grenby, T. H. & Young, L. (1959). *Biochem. J.* **71**, 25P.
Knight, R. H. & Young, L. (1958). *Biochem. J.* **70**, 111.
Krebs, H. A., Sykes, W. O. & Bartley, W. C. (1947). *Biochem. J.* **41**, 622.
Parke, D. V. & Williams, R. T. (1951). *Biochem. J.* **48**, xxvii.
Pirie, N. W. & Hele, T. S. (1933). *Biochem. J.* **27**, 1716.
Roberts, J. J. & Warwick, G. P. (1957). *Nature, Lond.*, **179**, 1181.
Roberts, J. J. & Warwick, G. P. (1958). *Biochem. Pharmacol.* **1**, 60.
Stoll, A. & Seebeck, E. (1948). *Helv. chim. acta*, **31**, 189.
Thomson, A. E. R., Maw, G. A. & Young, L. (1958). *Biochem. J.* **69**, 23P.

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The Effect of Growth on the Composition of Avian Muscle

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Muscular growth in birds and mammals is known to involve a fall in the percentage of water and extracellular electrolytes, and a rise in the percentage of protein, potassium and phosphorus (Leslie & Davidson, 1951; Robinson, 1952*a*; Barlow & Manery, 1954). McCance & Widdowson (1956) found that during the development of human and pig muscle there was an increase in the nitrogen/potassium ratio. This was interpreted to mean that development might be associated with a change in the gross composition of the muscle cell. The work on these two mammals has been extended and the results are reported elsewhere (Dickerson & Widdowson, 1960). The present paper describes observations of a similar nature on avian muscle.

EXPERIMENTAL

Birds used in this investigation were pure-bred Rhode-Island Red cockerels. Twenty-four birds were taken as soon as possible after they had hatched. A further 12 birds aged 2–3 weeks (wt. approx. 100 g.), nine aged 4 weeks (wt. approx. 200 g.) and four aged 27 weeks (wt. approx. 3·5 kg.) were used. The 27-week-old birds were assumed to be adult for the present purpose.

Methods

Newly hatched chicks. These were divided into three groups, each of eight animals, in such a way that the average weight of the birds in each group was approximately the same. The pectoral muscles of all the birds in each group were pooled for analysis. The birds aged 2–3 weeks and 4 weeks were also each divided into three groups and treated in a similar way. The muscle from each adult bird was analysed separately.

Young birds. These were killed by a blow on the head and about 1 ml. of blood was taken by cardiac puncture from each, and this clotted satisfactorily under liquid paraffin. The samples taken from the individual birds in each group were pooled so that three pooled samples of serum were obtained at each of these ages. The pectoral and sartorius muscles of each bird were dissected as completely as possible and placed in tared specimen tubes which had been previously cooled on solid CO_2. The frozen muscle was weighed.

Adult birds. These were killed by the injection of 180 mg. of pentobarbitone sodium (Nembutal). About 5 ml. of blood was taken by heart puncture before the animal died and allowed to clot under paraffin. Further haemorrhage was avoided (Widdowson & Southgate, 1959). The pectoral muscles of one side and the sartorius muscle of both sides were dissected as completely as possible, weighed at once and representative samples frozen on solid CO_2. The weight of the pectoral muscles of these animals was obtained by multiplying the weight taken from one side by two. A small piece of muscle from one animal of each age and kind was fixed in formalin–0·9% NaCl for histological examination. In preparation for analysis, the pectoral muscle was thawed, freed of visible fat and tendon, and thoroughly cut up with scissors.

Analyses

Proteins. These were separated by the method described by Robinson (1952*a*). Analyses were made on duplicate samples where possible. Each sample of 0·5–2·0 g. was weighed in a screw-capped Universal container (Baird and Tatlock Ltd.) which fitted the micro-attachment of a MSE homogenizer. The muscle samples were extracted in the homogenizer three times with 'dilute salt' solution (0·1 M-KCl, 0·066 M-NaH_2PO_4–K_2HPO_4, pH 7·1, *I* 0·2). The suspension was centrifuged between each extraction and the supernatant liquid removed. These extractions with salt solution were followed by two further extractions with 0·1 N-NaOH, after which the muscle samples were left to stand in 0·1 N-NaOH overnight at 4°. The 'dilute salt' and the NaOH extracts and the residue were retained. Actomyosin in the 'dilute salt' extract was precipitated by the addition of an equal volume of 30% (v/v) ethanol and separated in the centrifuge. The N in both the supernatant liquid and the precipitate was estimated. The former consisted of sarcoplasmic-protein N and non-protein N, and the sarcoplasmic-protein N was obtained by deducting the non-protein N obtained by separate estimation. The N in the extract with NaOH was estimated and this, together with the N in the actomyosin precipitate, constituted the fibrillar-protein N. The result for total fibrillar protein obtained in this way was in good agreement with that obtained by extraction of further samples with 'strong salt' solution (1·25 M-KCl, 0·066 M-K_2HPO_4, pH 8·5, *I* 1·45) followed by 0·1 N-NaOH as described by Robinson, and in some cases this further extraction was omitted. In all samples the 'fibrillar proteins' described in this and other work (Dickerson & McCance, 1960; Dickerson & Widdowson, 1960; Widdowson, Dickerson & McCance, 1960) correspond to the sum of Robinson's fibrillar and denatured fibrillar fractions. The residue after extraction with salt solution and NaOH has been assumed to be extracellular proteins (Lowry, Gilligan & Katersky, 1941). The sum of the fractions agreed with the total N within the limits ±5%.

Total protein nitrogen. This (actually protein + nucleic acid) was precipitated by the addition of 10 ml. of 10% (w/v) trichloroacetic acid to 0·5–1·0 g. of muscle. The muscle was snipped as finely as possible with scissors in the trichloroacetic acid and the suspension allowed to stand for at least 1 hr. After centrifuging, the supernatant liquid was removed and the precipitated protein extracted twice more with trichloroacetic acid solution. The supernatants were combined, the volume was measured and a sample was pipetted into a Kjeldahl flask for estimation of the non-protein N. The precipitated protein was dissolved in conc. H_2SO_4, made up to a known volume and a sample taken for estimation of N. All samples for estimation of N were digested with 2 ml. of conc. H_2SO_4 in the presence of copper selenite as catalyst. The N was subsequently estimated by micro-Kjeldahl distillation. The total N was either estimated independently or obtained by adding together the protein and non-protein N.

Collagen. This was estimated by direct determination of hydroxyproline in the hydrolysate of the mixed proteins of the tissue by the method of Neuman & Logan (1950). Skeletal muscle was dried to constant weight at 100°. The dry tissue was powdered and duplicate samples of 100–300 mg. were used for the estimation of collagen. The samples were hydrolysed with 6 N-HCl in sealed tubes in a boiling-water bath for 48 hr., 1 ml. of HCl/50 mg. of dry tissue being used. The subsequent procedure was the same as that described by Neuman & Logan (1950), except that 0·05 M-$CuSO_4$ solution was used as advocated by Baker, Lampitt & Brown (1953), and the extinction was read at a wavelength of 560 mμ. It was assumed that the collagen in fowl muscle contained 14·0% of hydroxyproline (Leach, 1957). No allowance was made for interference by tyrosine.

Water, chloride, sodium, potassium, phosphorus and magnesium. These were estimated by the same methods as those described elsewhere (Dickerson & Widdowson, 1960). The serum electrolytes were estimated as described by Widdowson & McCance (1956).

Chloride 'space'. This was calculated in the conventional way from the concentration of Cl in the muscle and that in the serum water. The Na 'space' has been calculated in a similar way. The Cl 'space' has been used as a measure of the proportion of extracellular fluid (Barlow & Manery, 1954). The concentrations of electrolytes found in the serum were similar to those reported by Barlow & Manery (1954) and these have been converted into concentrations in the extracellular fluid by applying factors of 1·05 for Cl and 0·94 for Na and K to make allowance for the Gibbs-Donnan equilibrium.

Changes in cell composition have been followed as described by Dickerson & Widdowson (1960), by using intracellular water and intracellular-protein N as reference standards for the cellular constituents.

RESULTS

Table 1 shows the effect of growth on the body weight and on the weight of the pectoral and sartorius muscles. The body weight of the chicks was almost doubled during the first 2½ weeks after hatching, but there was a tenfold increase in the weight of the pectoral muscles during this time. The sartorius muscles grew at a slower rate than the pectoral muscles and were always lighter. Only the pectoral muscles were chemically analysed.

Table 2 shows the effect of growth on the composition of skeletal muscle. Most of the fall in the amounts of water, Na and Cl/kg. of muscle took place during the first 2½ weeks after hatching. The large concentration of Na and the small concentration of K in the muscle of the chick at hatching agrees with the findings of Barlow & Manery (1954). During the first 2½ weeks the large fall in the concentration of Na was accompanied by an increase in the concentration of K. Potassium did not, however, continue to increase during further growth as

Table 1. *Effect of growth on the body weight and on the weight of the pectoral and sartorius muscles*

Average weights (g.) per bird are given.

Age (weeks) ...	0	2½	4	27
Body wt.	52·4	93	195	3365
Pectoral muscles	0·39	4·12	12·1	326
Sartorius muscles	0·13	0·28	0·53	13·5

Table 2. *Effect of growth on the composition of fowl pectoral muscle*

Amounts/kg. of fresh muscle are given as average and range.

Age (weeks)	0	2½	4	27
Number of analyses	3	3	3	4
Water (g.)	854 (847–864)	771 (768–774)	756 (753–760)	737 (724–740)
Total N (g.)	17·3 (16·5–18·1)	29·5 (28·4–30·5)	34·1 (33·1–35·5)	37·0 (33·8–38·9)
Non-protein N (g.)	1·7 (1·6–1·8)	4·2 (4·0–4·7)	4·5* (4·2, 4·9)	5·5 (5·0–6·1)
Total-protein N (g.)	15·6 (14·7–16·4)	25·3 (24·5–26·5)	29·5* (28·9, 30·1)	31·6 (28·8–33·5)
Sarcoplasmic-protein N (g.)	4·0 (3·4–4·6)	6·6 (6·5–7·4)	10·8* (10·6, 11·0)	11·1 (10·0–12·1)
Fibrillar-protein N (g.)	8·8 (8·4–9·0)	16·9 (16·7–17·1)	17·9* (17·3, 18·4)	19·4 (18·2–20·6)
Extracellular-protein N (g.)	2·8 (2·7–2·9)	1·6 (1·4–1·9)	1·1* (1·0, 1·2)	1·2 (1·0–1·4)
Collagen N (g.)	2·3 (2·0–2·5)	1·4 (all 1·4)	0·8 (all 0·8)	1·1 (0·8–1·4)
Cl (m-equiv.)	74·7 (71·0–80·1)	20·4 (18·8–21·8)	19·6 (19·4–19·7)	17·1 (13·6–19·0)
Na (m-equiv.)	137·3 (122·1–149·8)	19·3 (18·8–19·7)	20·1 (18·0–24·4)	19·9 (17·4–22·3)
K (m-equiv.)	46·0 (44·1–49·4)	124 (124–125)	116 (all 116)	104 (91–116)
P (m-moles)	53·9 (45·5–66·5)	87·8 (85·2–89·3)	82·3 (81·3–83·2)	80·0 (77·4–82·6)
Mg (m-equiv.)	23·2* (21·3, 25·0)	28·0 (26·9–29·5)	29·2* (27·7, 30·8)	28·8 (27·0–30·6)

* Two results only.

Table 3. *Effect of growth on the distribution of the total nitrogen*

Results are the amounts of N in the various fractions expressed as a percentage of the total N, and are the averages of the individual values.

Age (weeks)	0	2½	4	27
Non-protein N	9·9	14·2	13·4	14·8
Sarcoplasmic-protein N	22·8	22·9	31·7	30·0
Fibrillar-protein N	50·9	58·2	52·4	53·2
Extracellular-protein N	16·3	5·2	3·2	3·3
Collagen N	12·7	4·8	2·3	3·0

it does in human muscle (Dickerson & Widdowson, 1960), but steadily decreased to a somewhat lower value in the muscle of the adult. The concentration of total P changed in parallel with that of K.

The fall in the amount of water/kg. with development was accompanied by an increase in the amount of total N and, as with the water, most of the change took place in the first month of life. Parallel increases also took place in the non-protein N and in the total-protein N. The non-protein N was the nitrogen extracted from the muscle with trichloroacetic acid, and for this reason will include amino acids and polypeptides besides urea, creatine and purines.

The two groups of intracellular proteins, those of the sarcoplasm and fibrils, increased with development. The great increase in the fibrillar proteins took place in the first 2½ weeks. Between 2½ and 4 weeks they increased only a little more, whereas the sarcoplasmic proteins went on doing so. The amount of extracellular-protein N/kg. decreased rapidly after hatching and by 4 weeks had reached its adult amount. The changes in the amount of collagen N paralleled those of the total extracellular proteins, but, as collagen forms only a part of the latter, it was at all ages lower.

Table 3 shows the distribution of the N as a percentage of the total N. The non-protein N had almost reached its adult proportion of the total N by 2½ weeks, and by 4 weeks the composition of the pectoral muscle resembled that of an adult. The distribution of N amongst the various protein fractions is very similar to the pattern obtained by Robinson (1952*a*) at corresponding ages. The small differences may be due to the analysis of a different breed of bird.

Table 4 shows the distribution of the water and the relationship of intracellular-protein N, K, P and Mg to the intracellular water and of K, P and Mg to intracellular-protein N.

Table 4. *Effect of growth on the composition of avian skeletal muscle*

Data derived from average results shown in Table 2 for the distribution of water and the composition of the cells relative to cellular water and cellular-protein N.

Age (weeks)	0	2½	4	27
	Per kg. of muscle			
Chloride 'space' (g.)	550	161	144	124
Sodium 'space' (g.)	967	126	135	128
Intracellular water (g.)	304	610	612	613
Intracellular-protein N (g.)	12·8	23·7	28·4	30·4
Intracellular K (m-equiv.)	40·8	123	115	103
Intracellular Na (m-equiv.)	59·3	0	0	1·4
	Per kg. of intracellular water			
Intracellular-protein N (g.)	42·1	38·9	46·4	49·6
Intracellular potassium (m-equiv.)	134	202	188	168
Intracellular potassium plus intracellular sodium (m-equiv.)	330	202	188	168
P (m-moles)	177	144	135	130
Mg (m-equiv.)	26·6	38	40·1	39·5
	Per g. of intracellular-protein N			
Intracellular potassium (m-equiv.)	3·19	5·19	4·1	3·39
Intracellular potassium plus intracellular sodium (m-equiv.)	7·83	5·19	4·1	3·59
P (m-moles)	4·2	3·7	2·9	2·63
Mg (m-equiv.)	0·63	0·98	0·87	0·80

The changes in the Cl 'space' and intracellular water during growth were similar to those described by Barlow & Manery (1954) in fowl muscle and by a number of investigators (Yannet & Darrow, 1938; Hines & Knowlton, 1939; McCance & Widdowson, 1956; Dickerson & Widdowson, 1960) in mammalian muscle. The proportion of protein to liquid in the muscle cells decreased slightly during the first 2½ weeks of life but thereafter steadily increased. A similar sequence of events has been met with in the skeletal muscle of the pig and man (Dickerson & Widdowson, 1960). The concentration of K in the intracellular water moved in the opposite direction. The quantity of P steadily decreased whereas that of Mg decreased during the first 2½ weeks and then probably did not change further. The ratio of intracellular K to intracellular-protein N was lower at hatching than at 2½ weeks but from this time onwards decreased. The ratio of Mg to intracellular-protein N was higher at hatching than at 2½ weeks but changed little after this age. The ratio of P to intracellular-protein N decreased over the whole of the growth period. The large amount of Na in excess of that in the extracellular fluid was thought by Barlow & Manery (1954) possibly to be bound to solid in the extracellular phase, but if it was, in fact, inside the cells, then the ratios of intracellular Na plus intracellular K to intracellular water and to intracellular-protein N show a decrease over the whole of the growth period.

DISCUSSION

The pectoral muscles increased in weight some tenfold during the first 2½ weeks of life and this was associated with a decrease in the quantity of extracellular proteins in each kilogram of muscle. Similar changes have been found to take place in mammalian muscle (Dickerson & Widdowson, 1960) and have been attributed to an increase in the diameter of the muscle fibres. The rapid change in fowl muscle could hardly be attributed to this, however, for there was only a small increase in the diameter of the muscle fibres during the first 2½ weeks, though there was an increase during later life. Another reason which, however, may be put forward for these changes in the pectoral muscles is a change in shape of the muscle. At hatching, the pectoral muscle of the chick is a thin sheet of tissue with a relatively large surface area/volume ratio. Consequently, the thickest of its connective-tissue components, the epimysium, accounted for a large part of its extracellular proteins. During the first 2½ weeks, the muscle probably grew by increasing the number of fibres, and this growth took place at a faster rate than that of the bone underlying it. The result of this was that the muscle increased in thickness and the surface area/volume ratio fell.

It has been assumed in making some of the calculations shown in Table 4 that the intracellular water was evenly distributed throughout the cell.

This may not be so (Huxley & Hanson, 1957). Furthermore, skeletal-muscle cells contain intracellular structures which are common to all true cells. Of these, the nucleus is the most prominent and accounts for a larger proportion of the protein in the muscle cells of young animals (Robinson, 1952*b*), and it has been suggested that this might account for some of the differences in the composition of the muscle cell at different ages (Dickerson & Widdowson, 1960). It might, for example, partly account for the large amounts of sodium in the muscles of 'day-old' chicks and to a lesser extent in those of foetal mammals (Dickerson & Widdowson, 1960), for Itoh & Schwartz (1956) showed that the nuclei of liver and thymus contain more sodium than the cytoplasm and the same may be true of muscle nuclei.

The cells of chick-heart muscle at hatching also contain apparently more sodium than they do at later stages of growth (Dickerson, 1959). Oxygen is essential if the mechanism by which sodium is extruded from muscle cells is to work efficiently (Hercus, McDowall & Mendel, 1955) and it may be that the real explanation of the high sodium content of chick-muscle cells at hatching is to be found in the oxygen and energy supply of the muscles.

SUMMARY

1. Pectoral muscle from Rhode-Island Red cockerels at 0, 2½, 4 and 27 weeks of age were analysed for water, chloride, sodium, potassium, phosphorus and magnesium, and the total nitrogen was divided into non-protein, sarcoplasmic-, fibrillar- and extracellular-protein nitrogen.

2. The amounts of water, chloride, sodium and extracellular-protein nitrogen/kg. decreased and those of potassium, phosphorus and magnesium increased during the first 2½ weeks. Potassium and phosphorus fell from this age to a lower level in the adult. The fibrillar proteins increased mainly during the first 2½ weeks whereas the main increase in the sarcoplasmic proteins was between 2½ and 4 weeks. At 4 weeks the muscle had an almost adult composition.

3. Calculations showed that the increase in the proportion of the cellular phase was accompanied by an increase in the amount of protein nitrogen/l. of cell water, whereas the amount of sodium plus potassium/l. of cell water decreased.

4. The possible influence on cell composition of the uneven distribution of cellular constituents is briefly discussed.

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REFERENCES

Baker, L. C., Lampitt, L. H. & Brown, K. P. (1953). *J. Sci. Fd Agric.* **4**, 165.
Barlow, J. S. & Manery, J. F. (1954). *J. cell. comp. Physiol.* **43**, 165.
Dickerson, J. W. T. (1959). Ph.D. Thesis: University of Cambridge.
Dickerson, J. W. T. & McCance, R. A. (1960). *Brit. J. Nutr.* (in the Press).
Dickerson, J. W. T. & Widdowson, E. M. (1960). *Biochem. J.* **74**, 247.
Hercus, V. M., McDowall, R. J. S. & Mendel, D. (1955). *J. Physiol.* **129**, 177.
Hines, H. M. & Knowlton, G. C. (1939). *Proc. Soc. exp. Biol., N.Y.*, **42**, 133.
Huxley, H. & Hanson, J. (1957). *Biochim. biophys. Acta*, **23**, 229.
Itoh, S. & Schwartz, I. L. (1956). *Nature, Lond.*, **178**, 494.
Leach, A. A. (1957). *Biochem. J.* **67**, 83.
Leslie, I. M. & Davidson, J. N. (1951). *Biochim. biophys. Acta*, **7**, 413.
Lowry, O. H., Gilligan, D. R. & Katersky, E. M. (1941). *J. biol. Chem.* **139**, 795.
McCance, R. A. & Widdowson, E. M. (1956). *Quart. J. exp. Physiol.* **41**, 1.
Neuman, R. E. & Logan, M. A. (1950). *J. biol. Chem.* **184**, 299.
Robinson, D. S. (1952*a*). *Biochem. J.* **52**, 621.
Robinson, D. S. (1952*b*). *Biochem. J.* **52**, 628.
Widdowson, E. M., Dickerson, J. W. T. & McCance, R. A. (1960). *Brit. J. Nutr.* (in the Press).
Widdowson, E. M. & McCance, R. A. (1956). *Clin. Sci.* **15**, 361.
Widdowson, E. M. & Southgate, D. A. T. (1959). *Biochem. J.* **72**, 200.
Yannet, H. & Darrow, D. C. (1938). *J. biol. Chem.* **123**, 295.

Biochem. J. (1960) **75**, 38

Phosphorylated Compounds in the Head and Thoracic Tissues of the Adult Housefly, *Musca domestica* L., during Flight, Rest, Anoxia and Starvation

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The labelled-pool technique (Winteringham, 1956) has been used for studying the effects of insecticides on the relative concentrations of the soluble phosphorus compounds of intact adult houseflies. To facilitate the interpretation of the data so obtained it was also necessary to study the effects of physical activity, starvation etc. alone. These effects are now reported and discussed in the light of current ideas about carbohydrate metabolism and its regulation in insects.

Insects derive energy mainly by the oxidation of endogenous carbohydrate and fat, in that order, but during sustained flight Diptera appear to utilize only carbohydrate (Chadwick, 1947; Clements, 1955; Krogh & Weis-Fogh, 1951; Kubista, 1958; Sacktor, 1955; Wigglesworth, 1949; Zebe, 1954).

Carbohydrate catabolism and its regulation in insect muscle have recently received particular attention (Chefurka, 1958*a*; Silva, Doyle & Wang, 1958). Of considerable interest have been the observations that insect flight muscle contains remarkably high and low concentrations respectively of α-glycerophosphate dehydrogenase and lactic acid dehydrogenase (Sacktor, 1955; Zebe & McShan, 1957). There was little accumulation of lactic acid under conditions of anaerobic glycolysis; instead there was an accumulation of α-glycerophosphate and possibly of pyruvate and acetate (Barron & Tahmisian, 1948; Chefurka, 1958*a*; Humphrey, 1949; Kubista, 1957, 1958; Winteringham, 1958). No less than 20% of the total acid-soluble phosphorus in the thoracic tissues (mainly flight muscle) of the resting adult housefly appears in the form of α-glycerophosphate (Winteringham, 1959). Kubista (1957) and Chefurka (1958*b*) suggested that α-glycerophosphate dehydrogenase replaced lactic acid dehydrogenase in the oxidation of reduced coenzyme I in glycolysing insect muscle. Bücher and his colleagues (Bücher & Klingenberg, 1958) and, later, Sacktor (1958) have proposed a central role for α-glycerophosphate in the control of carbohydrate metabolism in insect flight muscle.

MATERIALS AND METHODS

Diphosphopyridine nucleotide and crystalline α-glycerophosphate dehydrogenase (from muscle). These were purchased from C. F. Boehringer und Söhne, Mannheim-Waldhof, Germany.

L-α-*Glycerophosphate.* The sodium salt was the kind gift of Dr R. W. Estabrook.

Insects and their treatment. Female adult houseflies (*Musca domestica* L.) not less than 3 days old at the time of extraction were used throughout. Unless stated otherwise in the tabulated results (below) the soluble phosphorus compounds were uniformly labelled with ^{32}P *in vivo*, as described earlier (Winteringham, Bridges & Hellyer, 1955), so that the specific radioactivity of the phosphorus of each compound was the same.

Extraction and paper-radiochromatographic analysis. After the soluble phosphorus pool had been labelled *in vivo*, groups of 10 adult female insects were normally immobilized by exposing them to *cyclo*propane for about 2 min., and were then plunged into liquid nitrogen to await dissection and extraction. The labelled compounds were extracted with ethanolic formic acid from the separated heads and thoraces (minus wing and leg appendages) with the slightly modified conditions already described (Winteringham, Hellyer & McKay, 1958). The extracted compounds were separated by chromatography and assayed radiometrically (Winteringham *et al.* 1955, 1958). Any special treatment of the insects before extraction is explained in the tabulated results below.

Chemical identity of the labelled fractions. Cochromatographic tests with authentic triose phosphates which had not been available in the earlier work strongly suggested that the compound described in the earlier papers (Winteringham *et al.* 1955, 1958) as phosphoglycerate was in fact α-glycerophosphate (Winteringham, 1959). This was confirmed enzymically as follows.

The oxidation of α-glycerophosphate (α-GP) in the presence of diphosphopyridine nucleotide (DPN) with the formation of dihydroxyacetone phosphate (DAP) and reduced DPN (DPNH) is specifically catalysed by muscle α-glycerophosphate dehydrogenase according to the equation

$$\text{DAP} + \text{DPNH} \rightleftharpoons \alpha\text{-GP} + \text{DPN}$$

with an equilibrium constant of $0{\cdot}17 \times 10^4$ at pH 8·1. (McShan, 1949; Beisenherz, Bücher & Garbade, 1955). In the present work 0·1 M-hydrazine sulphate as a trapping agent for DAP only changed the equilibrium constant to $2{\cdot}4 \times 10^2$ at pH 8·1. However, in the presence of hydrazine and a large excess of DPN a trace of [^{32}P]α-GP would thus be largely converted into the acid-labile [^{32}P]DAP.

The fraction behaving as [^{32}P]α-GP under the conditions of paper chromatography (Winteringham, 1959) equivalent to two thoraces was reconcentrated in a 1 mm. section of Whatman no. 1 paper (Winteringham, 1953) and dissolved in 0·5 ml. of water. On the basis of total soluble thoracic

phosphorus estimated by radioactivation (Winteringham *et al.* 1955), and the paper-radiochromatographic analysis of the particular extract used, 0·15 ml. of the above solution was equivalent to 0·013 μmole of possible α-GP. Samples of 0·15 ml. were diluted to 3 ml. with water (2·85 ml.) which also contained hydrazine sulphate (250 μmoles), DPN (10 μmoles) and active or heat-inactivated α-glycerophosphate dehydrogenase (0·4 mg.) at pH 8·1 (0·05 M-2-amino-2-hydroxymethylpropane-1:3-diol buffer). The formation of DPNH was followed spectrophotometrically at 340 mμ, and in the presence of the active enzyme alone reached the equivalent of 0·013 μmole of α-GP. This was in agreement with that estimated by paper-radiochromatographic and radioactivation analysis. The mixtures were finally acidified with conc. HCl and hydrolysed for 10 min. at 100°, and the hydrolysate was examined by paper-radiochromatography. Under these conditions any DAP formed enzymically would liberate its phosphorus as inorganic phosphate. The original ^{32}P behaved almost entirely as inorganic phosphate in the final radiochromatogram. These experiments thus confirmed that the fraction originally described as phosphoglycerate and representing some 20% of the total soluble thoracic phosphorus of the resting insect is L-α-glycerophosphate.

It is reiterated that in the tabulated results below the description of a ^{32}P-labelled fraction as a particular compound must not be taken to exclude the presence of other unidentified compounds in relatively small but possibly variable proportions. Fractions designated UC. 1, etc., are partly or completely resolved under the routine conditions of paper chromatography used but await identification. Recent work by the author's colleagues Mr R. G. Bridges and Mrs J. R. Beard has shown that the fast-running somewhat variable fraction earlier designated as UC. 4 (Winteringham *et al.* 1958) is almost certainly a soluble phospholipid fraction and is now so described in the tables. Up to 18% of the soluble thoracic ^{32}P has been extracted in this form from insects provided with dietary milk for 24–48 hr. preceding extraction.

The following abbreviations have been used in the tables and text: adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), glucose 6-phosphate (G 6-P), arginine phosphoric acid (Arg-P), inorganic phosphate (PO_4^{3-}), the phospholipid fraction recovered in ethanolic formic acid used for extraction (P lipid), unidentified phosphorus compounds (UC. 1, UC. 2 and UC. 3). Unresolved composite fractions have been indicated by brackets. Analyses of head and thoracic extracts from the same group of 10 insects is indicated by a common experiment number in the table.

RESULTS

Tabulation and significance of the data. The results are collected in Tables 1–6. The ^{32}P of each separated compound or fraction is expressed in units per cent of the total soluble ^{32}P recovered from the head (rich in nervous tissue, poor in muscle) or thorax (mainly flight muscle). Since the compounds are uniformly labelled, changes in the distributions of ^{32}P also represent changes in the relative tissue concentrations at the instant of chilling and extraction. As in the earlier work the distribution of phosphorus found in a single experiment has been taken as the mean of the two paper-radiochromatographic analyses made. The significance of differences between the results of single experiments and the means of several experiments has been estimated by the t test and corresponding levels of probability (P) are indicated. In applying the t test it has been assumed that the variance in experiments with treated insects would be the same as that found in the corresponding groups of control experiments and indicated by the standard errors of the means in Tables 1 and 2. The use of the angular transformation for testing the significance of differences between mean values of less than 15% (see Bliss & Calhoun, 1954) led to increased values of t. Its use was therefore abandoned as a precautionary measure.

Phosphorus compounds during rest. In Table 1 are the mean distributions of soluble phosphorus of the head tissues (11 groups of 10 flies of different generations) and of the thoracic tissues (49 groups of 10 flies representing 30 different generations). In all these experiments the insects were brought to a state of rest by the addition of *cyclo*propane to the air drawn through the metabolism chamber. An exposure of about 2 min. was usually adequate.

Phosphorus compounds during activity. In some experiments the insects were transferred to a small stainless-steel-gauze cage while under mild *cyclo*propane anaesthesia. They were then allowed to recover completely and were either standing or walking at the instant of plunging them into liquid nitrogen. The results are collected in Table 2. The only significant difference between the phosphorus distribution of the resting insect and that of the active insect was in the level of thoracic α-GP. The

Table 1. *Distribution of mean soluble phosphorus in the adult housefly at rest*

All insects were brought to rest by exposure to an air–*cyclo*propane mixture for about 2 min. before plunging them into liquid nitrogen and subjecting them to dissection, extraction, etc.

Fraction	^{32}P of fraction (% of total soluble ^{32}P recovered from head or thorax ± S.E.M.) Head (11 expts.)	Thorax (49 expts.)
ATP + UC. 1	35·9 ± 2·1	34·7 ± 0·69
ADP	> 4·9 ± 0·7	> 3·7 ± 0·34
AMP + UC. 2	4·3 ± 0·4	3·6 ± 0·27
G 6-P	10·6 ± 0·9	9·1 ± 0·46
UC. 3	0·8	Trace
Arg-P	6·2 ± 0·5	10·5 ± 0·49
α-GP	13·0 ± 1·2	20·9 ± 0·66
PO_4^{3-}	20·9 ± 1·3	16·8 ± 0·67
P lipid	3·4	0·7
	100	100

mean level of 20·9% of the resting insect fell significantly to 12·7% on resumption of visible activity ($P < 0·01$). As in other experiments these results also show that a significant change in the distribution of thoracic phosphorus was not invariably followed by a corresponding change in the head tissues. This may be due to the predominance of muscle- and nervous-tissue sources of phosphorus respectively in these two regions.

Phosphorus compounds during flight. Four experiments were made in which groups of individual insects were freely suspended by fine wires tied round the constriction between thorax and abdomen or held by suction applied to the dorsal thorax through a 1 mm. capillary. Momentary contact of the legs sometimes stimulated these insects into full captive flight. They were then immediately plunged into liquid nitrogen for extraction. The reluctance of some insects to attempt flight was apparently associated with their confinement to the relatively small glass metabolism chambers used or with the limited diet of aqueous glucose or both. In two experiments therefore the insects were transferred to a very large glass chamber and provided with milk, glucose and water *ad lib.* for several hours before the experiment. These insects had recovered their normal tendency to fly on stimulation. All the results are shown in Table 3. A consistent change which occurred on stimulating the non-anaesthetized insect into full flight was in the level of thoracic PO_4^{3-}, which rose significantly ($P < 0·01$) from a mean value of 20·4 (Table 2) to 28·0 (Table 3) during flight. Comparison of the data of Tables 2 and 3 suggest that the increase in PO_4^{3-} during flight was due to the breakdown of the composite fraction which contained Arg-P. No change in thoracic nucleotide levels or in any of the head compounds was indicated.

Phosphorus compounds during anoxia by drowning. Insects were transferred to stainless-steel-gauze cages while under *cyclo*propane anaesthesia and allowed to recover completely. They were then immersed in water containing a trace of Teepol wetting agent and were agitated to remove adhering air bubbles. In all cases struggles persisted for about 5 min. After different times the insects were quickly rinsed in acetone to remove superficial water and plunged into liquid nitrogen for extraction etc. The results are shown in Table 4.

The results suggest a rapid rise in the level of α-GP to a constant value of about 29% (mean level after 5 min. or more), which was significantly higher than that of the resting or active insect

Table 2. *Distribution of mean soluble phosphorus in the adult housefly during activity not involving flight*

No anaesthesia was used; all insects were standing, walking, etc., at the instant of death. In two experiments milk was added to the diet after labelling *in vivo*.

	^{32}P of fraction (% of total soluble ^{32}P recovered from head or thorax ± S.E.M.)	
Fraction	Head (7 expts.)	Thorax (9 expts.)
ATP + UC. 1	32·0 ± 2·6	32·2 ± 1·9
ADP	* 5·0 ± 0·7	* 4·7 ± 0·8
AMP + UC. 2	4·0 ± 0·9	2·5 ± 0·6
G 6-P	8·7 ± 1·0	8·3 ± 1·2
UC. 3	2·0	0·7
Arg-P	9·3 ± 2·5	13·4 ± 1·7
α-GP	13·0 ± 1·4	12·7 ± 1·3
PO_4^{3-}	21·7 ± 1·6	20·4 ± 1·7
P lipid	4·3	5·1
	100	100

Table 3. *Distribution of soluble phosphorus in the adult housefly during flight*

Insects were plunged into liquid nitrogen during captive flight. In Expts. 3 and 4 the insects were first conditioned by supplementary dietary milk and by the provision of adequate flying space.

	^{32}P of fraction (% of total soluble ^{32}P recovered from head or thorax)					
		Thorax				
Expt. no. ...	Head (4)	(1)	(2)	(3)	(4)	Mean values (thorax only)
Fraction						
ATP + UC. 1	38·7	51·6	28·9	37·2	34·8	41·0
ADP	* 4·2		* 5·5	0	* 6·1	
AMP + UC. 2	7·9	8·6	2·1	16·8	5·8	16·2
G 6-P			8·4			
UC. 3	Trace		0		0	
Arg-P	13·9		6·2		16·7	
α-GP	13·7	10·2	15·6	9·4	7·9	10·8
PO_4^{3-}	21·6	29·6	27·2	26·5	28·7	28·0
P lipid	Trace	0	6·1	10·1	Trace	4·0
						100

($P < 0{\cdot}01$). There was a delayed but obvious breakdown of the ATP + ADP fraction with the formation of PO_4^{3-}. Although barely significantly different from that of the resting insect ($0{\cdot}1 > P > 0{\cdot}05$) the high AMP value after 300 min. suggests that AMP is another product of nucleotide breakdown. The level of Arg-P after 300 min. was significantly lower than that of the resting insect ($P < 0{\cdot}05$). Thus extended anoxia caused a drastic breakdown of high-energy-phosphorus compounds but this breakdown might have been a post-mortem change.

Phosphorus compounds during starvation. A common effect of insecticides in adult houseflies is loss of locomotive ability and co-ordination so that the insect is unable to feed or drink, exhausts its endogenous food and becomes desiccated. Experiments were therefore made to determine the effects of starvation alone. The results are summarized in Table 5.

It will be seen that, provided that the insects were behaving normally up to the time of extraction, starvation and the contingent desiccation had no effect. The distributions of head and thoracic phosphorus of Expt. 1 were not significantly different from those of the normal resting insect (Table 1). At the stage of prostration (Expt. 2), however, there was a significant fall ($P < 0{\cdot}01$) in the head ATP, the phosphorus appearing as PO_4^{3-}. That this was not some degenerative change associated with the onset of death was shown by its complete reversal by glucose injection and recovery of the insect (Expt. 3). Expt. 2 also demonstrated that changes in the head and thoracic tissues may occur independently (see above) because there was no significant change in thoracic phosphorus at prostration. Expts. 2 and 3 may be compared with those of Table 2 because the insects were not immobilized with *cyclo*propane.

Expt. 4 shows that when the condition of the prostrated insects had deteriorated to the point where glucose therapy was ineffective (1 hr. or more after becoming prostrate) there was an obvious breakdown in ATP and accumulation of PO_4^{3-} in both head and thoracic tissues.

Phosphorus compounds under various conditions. It was possible that the difference in thoracic α-GP levels in the so-called resting insect (Table 1) and in

Table 4. *Effects of drowning on the distribution of thoracic soluble phosphorus in the adult housefly*

Non-anaesthetized insects were submerged in air-free water containing a wetting agent, for different times, before extraction.

	^{32}P of fraction (% of total soluble ^{32}P recovered from the thorax)		
Expt. no.	(1)	(2)	(3)
Duration of anoxia (min.)	5	120	300
Fraction			
ATP + UC. 1	30·7	3·9	5·9
ADP			
AMP + UC. 2	13·3	16·6	7·0
G 6-P			5·0
UC. 3			0
Arg-P			2·8
α-GP	26·9	32·2	27·7
PO_4^{3-}	29·1	42·1	49·2
P lipid	0	5·2	2·4

Table 5. *Effects of starvation on the distribution of soluble phosphorus of head and thorax in the adult housefly*

Expt. 1. Insects were deprived of food and water for 5–10 hr. and finally were anaesthetized with *cyclo*propane, plunged into liquid nitrogen etc. All insects behaved normally up to the time of extraction. *Expt.* 2. Insects were starved to the point of complete prostration (10–12 hr.: on backs, no wing or leg movements). Individual insects were transferred to liquid nitrogen without anaesthesia immediately after becoming prostrate. *Expt.* 3. Exactly as in (2) but prostrate insects were intrathoracically injected with aqueous glucose (200 μg.). They were transferred to liquid nitrogen 1 hr. after injection of glucose, when all the insects had recovered and were behaving normally. *Expt.* 4. Insects were starved to death (20–24 hr.; no recovery on glucose injection).

	^{32}P of fraction (% of total soluble ^{32}P recovered from the head or thorax)							
	Head				Thorax			
Expt. no. ...	(1)	(2)	(3)	(4)	(1)	(2)	(3)	(4)
Fraction								
ATP + UC. 1	35·9	14·8	28·4	9·4	29·9	27·8	28·1	11·9
ADP	⁎4·7	⁎5·8	⁎8·6	⁎2·6	⁎6·8	⁎4·6	⁎6·1	⁎3·5
AMP + UC. 2		3·8	7·4	3·9	2·4			4·9
G 6-P	22·2	14·8	11·0	7·0	8·8	30·4	27·0	7·5
UC. 3		Trace	Trace	4·1	0·7			0·6
Arg-P		3·7	5·1	3·7	12·4			3·6
α-GP	11·0	8·2	12·4	8·5	21·8	11·1	11·7	5·9
PO_4^{3-}	21·1	42·4	19·1	49·1	14·8	21·5	17·1	53·9
P lipid	5·1	6·5	8·0	11·7	2·4	4·6	10·0	8·2

the active insect (Table 2) was not due to a fall in α-GP during activity but due to an accumulation as a result of some pharmacological or toxic effect (e.g. anoxia) of the anaesthetic itself. This seemed unlikely, however, because if the insect were deeply anaesthetized for more than 3 hr. by exposure to air–*cyclo*propane mixture there was no further change in head or thoracic phosphorus distribution beyond that which occurred on cessation of activity after 2 min. (cf. Expt. 1 of Table 6 with the results of Table 1). Similarly, deep anaesthesia with 1:2-dichloroethane vapour had no significant effect on the α-GP level (Expt. 2). It has already been shown that under conditions of anoxia there is a marked further rise in thoracic α-GP (Table 4).

In studying the effects of insecticides these were invariably applied topically in acetone solution to the dorsal surface of the thorax or were injected intrathoracically in water or acetone while the insect was under mild *cyclo*propane anaesthesia. For this purpose an Agla micrometer syringe (Burroughs Wellcome and Co., London) fitted with a 27 S.W.G. needle was always used. Numerous experiments have shown that provided that the insect was not injured (as shown by its rapid recovery of normal activity) there was no significant effect on phosphorus distribution. The results of Expts. 3 and 4 of Table 6 are typical. Insects which showed any sign of injury as a result of treatment or handling were never used in the preparation of extracts. Other experiments (Winteringham *et al.* 1955) have shown that extension of the metabolism time from 24 to 120 hr. had no effect on thoracic phosphorus distribution. Higher but variable proportions of ^{32}P were sometimes found in the P-lipid fractions of the thoraces of insects supplied with dietary milk (mean value 8·9 %), but there were no effects on the distribution of ^{32}P between the remaining fractions.

DISCUSSION

Metabolic regulation during flight. There is evidence that in Diptera the blood trehalose provides an immediate carbohydrate reserve for the flying insect, that it is broken down as far as pyruvate through the classical Embden–Meyerhof pathway, and oxidized by means of the Krebs cycle (Chefurka, 1958*a*; Evans & Dethier, 1957; Winteringham, 1957, 1958). The assumption of full flight leads to a dramatic increase in respiration (Sacktor, 1958) and this in turn suggests critical changes at certain rate-limiting steps of oxidative metabolism. A much simplified but likely scheme of trehalose catabolism and associated ATP synthesis is illustrated in Fig. 1. A remarkably high rate of α-GP oxidation through a structurally bound DPN-independent oxidase (Fig. 1, reaction 5) from locust-wing muscle led Bücher and his colleagues (Bücher & Klingenberg, 1958) to postulate an α-GP cycle (see Fig. 1). In the conventional mechanism of respiratory control (Lardy, 1955), ADP and PO_4^{3-} arising from an energy-dissipating process such as the rapid contractions of flight muscle provide the necessary substrates for oxidative phosphorylation and the contingent flow of electrons through the respiratory chain. In the postulated α-GP cycle this would permit the rapid oxidation of α-GP by the structurally-bound DPN-independent oxidase shown as reaction 5 (Fig. 1).

Table 6. *Effects of extended anaesthesia, acetone and water injections on distribution of soluble phosphorus in the adult housefly*

Expt. 1. Insects were deeply anaesthetized by continuous exposure to an air–*cyclo*propane mixture for 3·5 hr. and finally were removed to fresh air for about 30 min. until there were slight movements, to ensure that dead insects were not included. *Expt.* 2. Deep anaesthesia was induced by exposure to 1:2-dichloroethane vapour–air mixture for 6 min. *Expt.* 3. Flies were anaesthetized and extracted 2 hr. after injection of 1 μl. of acetone. *Expt.* 4. Flies were anaesthetized and extracted 2 hr. after injection of 2 μl. of water. Insects were allowed 48 hr. metabolism after labelling *in vivo*.

	^{32}P of fraction (% of total soluble ^{32}P recovered from the head or thorax)					
	Head		Thorax			
Expt. no. ...	(1)	(4)	(1)	(2)	(3)	(4)
Fraction						
ATP + UC. 1	37·3	31·9	30·6	35·1	36·7	30·5
ADP	≯2·2		≯2·0	0	0	≯3·7
AMP + UC. 2				4·0	0	1·8
G 6-P	27·0	22·9	33·0	9·0	11·6	6·5
UC. 3				0	0	0
Arg-P				5·6	13·5	19·0
α-GP	14·4	17·6	17·0	15·9	15·1	15·1
PO_4^{3-}	19·1	19·4	17·4	30·4	23·1	23·4
P lipid	Trace	8·2	Trace	0	Trace	0

Reoxidation of DPNH arising glycolytically through reaction 6 would follow by the action of the DPN-dependent α-GP dehydrogenase of the sarcoplasm (reaction 8) and glycolysis could so proceed. Bücher & Klingenberg (1958) obtained a mitochondrial preparation from locust-wing muscle which displayed respiratory control by ADP with α-GP as substrate. These workers further suggested that flavoprotein was the hydrogen acceptor so that oxidative phosphorylation could proceed with a theoretical maximal P:O ratio of 2. On the other hand Sacktor and his colleagues (Sacktor & Cochran, 1958; Chance & Sacktor, 1958; Estabrook & Sacktor, 1958*a*, *b*), using a thoracic mitochondrial preparation from houseflies, were unable to demonstrate respiratory control by ADP. They found slight respiratory stimulation with dibromophenol but not with dinitrophenol. Their α-GP-oxidase preparation required the presence of bivalent cations such as Mg^{2+} and was inhibited by chelating agents such as ethylenediaminetetra-acetate. These observations led them to suggest a

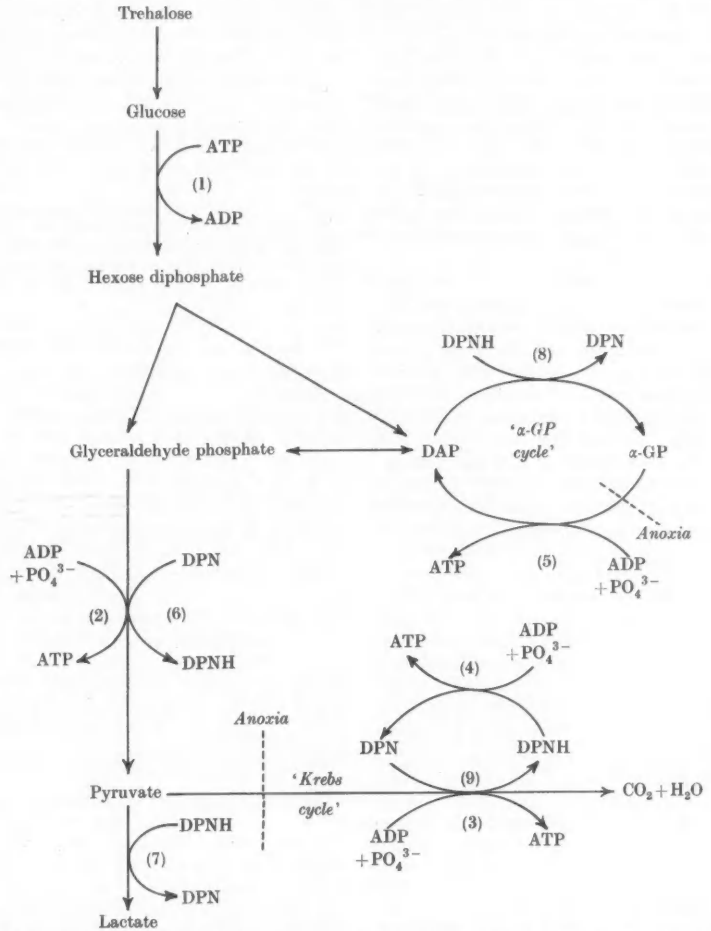

Fig. 1. Simplified scheme of carbohydrate catabolism in insect flight muscle, showing substrate-linked and oxidative phosphorylation, alternative DPN cycles and the steps affected by anoxia. (1) Substrate-linked dephosphorylation; (2), (3), substrate-linked phosphorylation; (4), (5), respiratory chain-coupled oxidative phosphorylation in mitochondria; (6), (7), (8), DPN-linked dehydrogenases in sarcoplasm; (9), DPN-linked dehydrogenases in mitochondria or sarcoplasm.

mechanism of control of α-GP-oxidase activity by an endogenous chelating agent in the flight muscle: 'During rest, glycerol-3-P oxidase is in an inhibited state... in flight, the inhibition is reversed and this brings about the high respiratory rate characteristic of the glycerol-3-P cycle.' Whether the α-GP cycle is under respiratory control as suggested by Bücher or controlled by the action of an endogenous chelating agent as suggested by Sacktor it will be seen that in either event α-GP might be expected to accumulate during rest and to become depleted during flight. The present work has shown that the level of α-GP fell significantly in the transition from *cyclo*propane-induced rest to the activity associated with walking or standing, but there was no further significant fall on the assumption of full flight. There was, however, a significant rise in the level of free PO_4^{3-} and this would be consistent with respiratory control in the intact flying insect. The methods used were probably insufficiently sensitive to detect small increases in amounts of phosphate acceptors such as ADP, although the fall in the fraction containing arginine phosphoric acid suggests that free arginine may also have increased during flight.

Effects of anoxia. If, in insect flight muscle, the DPN-dependent α-GP dehydrogenase (Fig. 1, reaction 8) replaces lactic acid dehydrogenase (reaction 7) in the reoxidation of DPNH during anaerobic glycolysis, an accumulation of α-GP would be expected under the same conditions because reaction 5 would be inoperative and reaction 7 absent. Kubista (1957) has demonstrated such an accumulation in the isolated thoracic muscles of the cockroach *Periplaneta americana*. A similar accumulation has now been found in the muscle of the intact housefly but, unlike the results of Kubista, there was also an increase in inorganic phosphate as a result of the breakdown of high-energy phosphates.

Effects of SH reagents. In a previous paper (Winteringham *et al.* 1958) it was reported that iodoacetate or excessive doses of methyl bromide vapour caused a fall in the phosphoglycerate level of adult houseflies. This effect should now be described as a fall in α-GP, which, however, would still be consistent with the inhibition of triose phosphate dehydrogenase *in vivo*. Inhibition of the SH-dependent triose phosphate dehydrogenase (Fig. 1, reaction 6) would reduce the supply of DPNH for reaction 8. Unless reaction 5 were equally sensitive to the SH reagent a depletion of α-GP would then occur.

SUMMARY

1. The presence of α-glycerophosphate representing some 20% of the total soluble phosphorus of the thoracic tissues of the resting intact adult housefly has been confirmed enzymically.

2. The transition from *cyclo*propane-induced rest to normal activity was associated with a fall in thoracic α-glycerophosphate. A further fall on the assumption of full flight was not apparent but the level of inorganic phosphate rose significantly.

3. Anoxia due to drowning caused an accumulation of thoracic α-glycerophosphate and a slower breakdown of adenosine triphosphate with the formation of inorganic phosphate and possibly adenosine monophosphate.

4. Starvation to the point of prostration caused a fall in head adenosine triphosphate which could be reversed by injection of aqueous glucose.

5. Extended *cyclo*propane anaesthesia, injected water or acetone were apparently without effect on phosphorus distribution.

6. Changes in head and thoracic phosphorus compounds could occur independently in the same insect.

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REFERENCES

Barron, E. S. G. & Tahmisian, T. N. (1948). *J. cell. comp. Physiol.* **32**, 57.

Beisenherz, G., Bücher, T. & Garbade, K-H. (1955). In *Methods of Enzymology*, vol. 1, p. 391. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Bliss, C. I. & Calhoun, D. W. (1954). *An Outline of Biometry*, p. 238. New Haven, Conn.: Yale Co-operative Corporation.

Bücher, T. & Klingenberg, M. (1958). *Angew. Chem.* **70**, 552.

Chadwick, L. E. (1947). *Biol. Bull., Woods Hole*, **93**, 229.

Chance, B. & Sacktor, B. (1958). *Arch. Biochem. Biophys.* **76**, 509.

Chefurka, W. (1958*a*). *Symp. 4th int. Congr. Biochem., Vienna*, **12**, no. 6.

Chefurka, W. (1958*b*). *Biochim. biophys. Acta*, **28**, 660.

Clements, A. N. (1955). *J. exp. Biol.* **32**, 547.

Estabrook, R. W. & Sacktor, B. (1958*a*). *Arch. Biochem. Biophys.* **76**, 532.

Estabrook, R. W. & Sacktor, B. (1958*b*). *J. biol. Chem.* **233**, 1014.

Evans, D. R. & Dethier, V. G. (1957). *J. Insect Physiol.* **1**, 3.

Humphrey, G. F. (1949). *J. cell. comp. Physiol.* **34**, 323.

Krogh, A. & Weis-Fogh, T. (1951). *J. exp. Biol.* **28**, 344.

Kubista, V. (1957). *Nature, Lond.*, **180**, 549.

Kubista, V. (1958). *Abstr. 4th int. Congr. Biochem., Vienna*, p. 149.

Lardy, H. A. (1955). *Conf. Rapports 3ème Congr. int. Biochem., Bruxelles*, p. 287.

McShan, W. H. (1949). In *Respiratory Enzymes*, p. 133. Ed. by Lardy, H. A. Minneapolis: Burgess Publishing Co.

Sacktor, B. (1955). *J. biophys. biochem. Cyt.* **1**, 1.
Sacktor, B. (1958). *Symp. 4th int. Congr. Biochem., Vienna*, **12**, no. 7.
Sacktor, B. & Cochran, D. G. (1958). *Arch. Biochem. Biophys.* **74**, 266.
Silva, G. M., Doyle, W. P. & Wang, C. H. (1958). *Nature, Lond.*, **182**, 102.
Wigglesworth, V. B. (1949). *J. exp. Biol.* **26**, 150.
Winteringham, F. P. W. (1953). *Nature, Lond.*, **172**, 727.
Winteringham, F. P. W. (1956). *Int. J. appl. Radiat. Isotopes*, **1**, 57.
Winteringham, F. P. W. (1957). *Chem. & Ind.* p. 1195.
Winteringham, F. P. W. (1958). *Symp. 4th int. Congr. Biochem., Vienna*, **12**, no. 5.
Winteringham, F. P. W. (1959). *Biochem. J.* **71**, 21P.
Winteringham, F. P. W., Bridges, P. M. & Hellyer, G. C. (1955). *Biochem. J.* **59**, 13.
Winteringham, F. P. W., Hellyer, G. C. & McKay, M. A. (1958). *Biochem. J.* **69**, 640.
Zebe, E. C. (1954). *Z. vergl. Physiol.* **36**, 290.
Zebe, E. C. & McShan, W. H. (1957). *J. gen. Physiol.* **40**, 779.

Biochem. J. (1960) **75**, 45

A Hydrolytic Procedure for the Identification and Estimation of Individual Phospholipids in Biological Samples

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Procedures which have been developed for the assay of individual phospholipids within a mixed-lipid sample isolated from tissues, usually depend upon a total hydrolysis of the phospholipids followed by separate determinations of the hydrolysis products, e.g. choline, phosphorus, inositol, serine, ethanolamine and sphingosine. Not only does this require the setting up of a large number of individual time-consuming assays, but the method suffers from the disadvantage that it fails to distinguish between the plasmalogens and the usual di-acylated phospholipids, e.g. between choline plasmalogen and lecithin.

The present method is based on one developed some years ago for the examination of phospholipid fractions by paper chromatography after mild alkaline hydrolysis (Dawson, 1954*a*). Such a hydrolysis results in the removal of the fatty acids from the alkali-labile phospholipids, leaving recognizable water-soluble fragments (mainly the corresponding esters of glycerophosphoric acid, e.g. glycerylphosphorylcholine) which can be subsequently separated and estimated by paper chromatography. This procedure has been simplified so that quantitative recoveries of the deacylated phospholipid fragments can be achieved, and extended so that the alkali-stable phospholipids (defined in this paper as the phosphorus remaining soluble in lipid solvent after alkaline hydrolysis) can also be examined. Thus the alkali-stable phospholipids are subjected to mild acid hydrolysis when the deacylated plasmalogens break down, giving a long-chain aldehyde and their corresponding water-soluble phosphodiesters. Finally the residual phospholipids are hydrolysed in methanolic hydrochloric acid, when sphingomyelin gives phosphorylcholine and sphingosylphosphorylcholine (Rennkamp, 1949; Dawson, 1958*a*).

The method requires the use of only a single assay procedure (for phosphorus) and it is possible to resolve and assay complex lipid samples within 2 days.

EXPERIMENTAL

Preparation of lipid samples

The procedure of Folch, Lees & Sloane-Stanley (1957) was mainly followed. The tissue samples were homogenized in 24 vol. of $CHCl_3$–methanol (2:1, v/v) in a high-speed blender. After standing for 30 min. with stirring, the suspension was centrifuged and filtered. The filtrate was shaken for 2–3 min. with 0·2 vol. of 0·9% NaCl soln. After centrifuging the emulsion, or leaving it overnight at 0°, the upper aqueous layer containing any water-soluble phosphorus impurities was removed and discarded. The lower layer was taken to dryness *in vacuo*, dissolved in an equal volume of $CHCl_3$, analysed for total phosphorus and stored at −20° until required.

As the subsequent alkaline hydrolysis of the phospholipids was performed with a limited amount of alkali, the method was not directly applicable to lipid extracts containing large amounts of neutral fat and low concentrations of phospholipid (< 40%, w/w), e.g. extracts of adipose tissue or fatty livers. With such extracts it is first necessary to separate the phospholipids with a simple silicic acid column or by other means (Borgström, 1952; Barron & Hanahan, 1958).

Alkaline hydrolysis of the phospholipids

Method I. The lipid sample, containing about 500 μg. of lipid P (not more than 550 μg.) was pipetted into a 50 ml. round-bottomed flask with a ground-glass joint fitted with a standard splash head. The solvent was removed at 40–50° *in vacuo* with constant swirling to avoid splashing. The residue was redissolved in 0·8 ml. of A.R. carbon tetrachloride (ethanol, methanol or light petroleum can also be used). It was found essential to use pure carbon tetrachloride as some technical grades gave pink spots on the subsequently developed chromatograms. Ethanol (7·5 ml.) was then added, followed by 0·65 ml. of water and 0·25 ml. of aq. N-NaOH. The mixture was incubated at 37° for 20 min. and the pH tested with indicator paper. The hydrolysate was still distinctly alkaline, provided that the lipid sample used did not contain more than 550 μg. of lipid P or excess of esterified neutral fat. Ethyl formate (0·4 ml.) was then added and, after mixing, the solution was incubated for a further 5 min. at 37°. This neutralized excess of alkali by the reaction: ethyl formate + NaOH = sodium formate + ethanol. The hydrolysate was then taken to dryness *in vacuo* below 60°. A volume of water was equilibrated with 2 vol. of *iso*butanol–$CHCl_3$ mixture (1:2, v/v), by shaking for a few minutes and allowing to settle. Samples from the upper aqueous layer (1 ml.) and lower solvent layer (2 ml.) were added to the dry residue of the hydrolysate, and the flask was shaken and warmed slightly to ensure the quantitative solubilization of the hydrolysis products and the alkali-stable phospholipids. The emulsion was then transferred as completely as possible to a centrifuge tube. After centrifuging, the upper aqueous layer (A) was withdrawn with a Pasteur pipette and stored for chromatography at 0°.

Method II. The hydrolysis was conducted in the same way as in method I. However, alkali was removed by passing the hydrolysate cooled to 0° through a short column of Amberlite IRC 50 H^+ (3 cm. × 0·8 cm. bore). The hydrolysis products were washed through the column with two successive 5 ml. portions of 80% ethanol. The total effluent of the column was taken to dryness *in vacuo* and then distributed between water and *iso*butanol–$CHCl_3$ as in method I. The water-soluble phosphorus-containing hydrolysis products passed quantitatively through the resin column but the alkali-stable phospholipids were partially adsorbed. For this reason the resin columns were never regenerated and method II could not be used for the subsequent examination of the alkali-stable phospholipids.

Hydrolysis of the plasmalogens

From the slightly turbid lower solvent layer containing the alkali-stable phospholipids, a sample (1·6 ml.) was withdrawn with a pipette operated by a hand-controlled suction bulb. To ensure that this fraction was not contaminated with any of the aqueous layer, the point of the pipette was inserted below the interface and any entrapped water driven out by slightly warming the pipette with the hand. To the sample contained in a 10 ml. stoppered centrifuge tube was added 0·8 ml. of 10% (w/v) trichloroacetic acid solution. The mixture was vigorously shaken (mechanically) for 30 min. in a water bath at 37°. On cooling, 2 ml. of light petroleum (b.p. 40–60°) was added and, after shaking, the tube was centrifuged. The upper solvent layer containing the alkali- and acid-stable phospholipids was removed as completely as possible with a Pasteur pipette. The lower aqueous layer containing the hydrolysed plasmalogen phosphorus was washed twice in the centrifuge with 5 ml. of $CHCl_3$–ether (1:4, v/v). This removed the remaining alkali- and acid-stable phospholipids and also the trichloroacetic acid. The aqueous layer was then adjusted to a neutral or slightly alkaline pH by holding a wick soaked in dilute ammonia solution in the air of the tube for a few seconds, which was then stoppered and shaken. It was stored at 0° for chromatography (B).

Hydrolysis of phospholipids stable to mild alkaline and acid hydrolysis

The combined solvent layers containing the alkali- and acid-stable phospholipids were taken to near dryness *in vacuo*. This operation was conveniently performed by evaporating successive small portions at 100°, in a drawn-out thick-walled test tube (Fig. 1). The final solvent removal was effected by inserting the end of a thick-walled piece of pressure tubing against the tapering neck of the tube and applying a vacuum (Fig. 1). To the residue of lipids and trichloroacetic acid was added 1·25 ml. of dry methanolic 1·7 N-HCl. The tube was sealed off in ice and then left in an oven at 100–102° for at least 4 hr. The hydrolysis tube was shielded as a precaution against a pressure explosion and

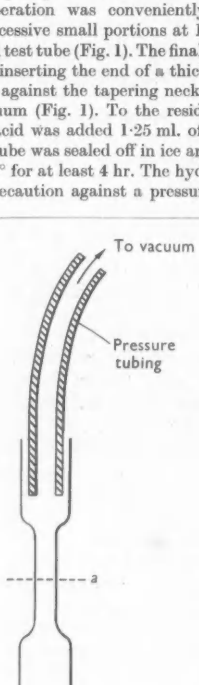

Fig. 1. Arrangement for evaporation of solvent from hydrolysis tube.

cooled well before opening at line '*a*' (Fig. 1). The pressure tubing was now applied over the tapering neck and the methanolic HCl removed *in vacuo*. To the hydrolysed lipids and residual methyl trichloroacetate (0·05 ml.) was added 0·45 ml. of 80% ethanol (C). (Some commercial samples of trichloroacetic acid give residues greater than 0·05 ml. and should be rejected.)

Paper chromatography and ionophoresis

Samples from the three hydrolysates were quantitatively spotted with an Ostwald pipette onto Whatman no. 1 paper which had previously been well washed with 2N-acetic acid and water. Spots were confined to an area of about 1·5–2·0 cm. diameter.

With the hydrolysate of the alkali-labile phospholipids (A) a 0·2 ml. sample was used, and a descending or ascending chromatogram was developed for 14–18 hr. with phenol saturated with water–acetic acid–ethanol (100:10:12, by vol.). For successful chromatography it was found essential to prepare the solvent freshly for each run. If the chromatogram was to be developed in a second dimension it was allowed to dry at room temperature and then washed twice with technical ether to remove phenol. The second solvent used was freshly prepared: methanol–98% formic acid–water (80:13:7, by vol.; ascending chromatography, 8 hr.); alternatively, separation in the second dimension was carried out by ionophoresis. Volatile buffer at pH 3·6 (pyridine–acetic acid–water; 1:10:89, by vol.) was applied to the paper and the phosphate esters were separated by ionophoresis at 2000 v (55 min.) under water-cooled toluene in an apparatus similar to that described by Ryle, Sanger, Smith & Kitai (1955).

The phosphate esters in a 0·3 ml. sample of the hydrolysate of the plasmalogens (B) were separated on a unidimensional paper chromatogram with either the same phenol–acetic acid–ethanol solvent or phenol saturated with water. The hydrolysate of the phospholipids which were stable to mild alkaline and acid hydrolysis (0·2 ml., C) was chromatographed with phenol saturated with water. After unidimensional chromatography the chromatograms were dried in an oven at 60–75°.

Detection of phosphorus-containing spots

A preliminary spray of the dried chromatograms with 0·25% of ninhydrin in acetone followed by heating at 100° for 3 min. assisted in the location of glycerylphosphorylethanolamine and its serine analogue. However, this was not used in the routine analytical procedure. The phosphorus-containing hydrolysis products were located by using the acid molybdate spray of Hanes & Isherwood (1949) followed by irradiation with u.v. light. However, for alkaline hydrolysates containing sodium formate (method I) the spraying reagent was modified and contained 10 ml. of 72% (w/w) perchloric acid, 20 ml. of 5N-HCl, 40 ml. of 5% ammonium molybdate solution and 130 ml. of water. Although this results in slower development of the spots it largely prevents the appearance on the chromatogram of a large greenish blue spot due to the alkali metal. After spraying, the chromatogram was thoroughly dried in a stream of air. The spots were then developed by exposure to a strong source of u.v. (a Hanovia Chromatolite u.v. lamp with the glass filter removed). Very slow development of the spots was found to be due to too much acid remaining in the paper from the acid molybdate spraying.

Estimation of phosphorus in spots

Each spot, plus a generous 0·5 cm. border, was cut out of the chromatogram, weighed on a torsion balance and introduced into a Pyrex boiling tube. Perchloric acid (72%, w/w) was added, the amount depending on the weight of paper spot to be oxidized (1–2 ml.). The tube was heated on an electrical digestion rack suitably protected against possible explosions (many thousands of such oxidations have been performed in this Laboratory with no such occurrence). If 72% perchloric acid was used (but not with 60%) the digestion fluid became colourless within 5–10 min. the oxidation being catalysed by the molybdate in the paper. At this stage the perchloric acid was evaporated by strong heating and reduced in volume as judged by eye to 0·6–0·7 ml. The estimation of phosphorus is independent of perchloric acid volumes between 0·5 and 0·9 ml. When cool the digestion fluid was quantitatively transferred to a tube graduated at 10 ml., with about 8 ml. of water. If a slight precipitate was present, this was removed by heating the tube for 10 min. at 100°. The tube was then cooled to room temperature [the colour development is dependent on temperature (Allen, 1940)]; 0·5 ml. of 5% ammonium molybdate was added and, after mixing, 0·4 ml. of reducing reagent (Fiske & Subbarow, 1925). The solution was made up to the 10 ml. mark, mixed and allowed to stand for 20 min. and the colour read at 660 mμ. Suitable weighed paper blanks were used, and the actual blank for each spot was calculated from its weight compared with the weight and colour reading of the blank: such blanks were usually low (E 0·01–0·03).

Calculation of phospholipid concentrations

For the samples and dilutions used in the above procedure the following calculations were applied to determine the percentage of phosphorus from a given phospholipid in the total phospholipid mixture. If P_T is the total phospholipid phosphorus hydrolysed and P_X the phosphorus in the spot being considered, the percentage of an alkali-labile phospholipid

$$=\frac{P_X}{P_T}\times\frac{1}{0{\cdot}2}\times 100.$$

For some phospholipids in this group it was necessary to apply a correction to allow for the further breakdown of the deacylated phospholipid derivatives. Thus certain of the glycerylphosphoryl derivatives can be partially broken down during alkaline hydrolysis to form cyclic glycerophosphoric acid (Maruo & Benson, 1959). Under the conditions of alkaline hydrolysis used, about 6% of the glycerylphosphorylcholine formed from lecithin was converted into the cyclic glycerophosphoric acid (6·1, 5·2, 6·1%), so the phosphorus found in the glycerylphosphorylcholine spot was multiplied by 1·06. Similarly, the phosphorus of the combined glycerylphosphorylinositol and phosphorylinositol spot derived from monophosphoinositide was multiplied by 1·40 to allow for the cyclic glycerophosphoric acid and other phosphorus-containing hydrolysis products also formed (see below). On unidimensional chromatograms the glycerylphosphorylethanolamine spot ran to the same position as the cyclic glycerophosphoric acid spot (Fig. 2). Consequently, a correction was made by subtracting the calculated amount of cyclic glycerophosphoric acid formed by the hydrolysis of lecithin and monophosphoinositide

present, i.e. 6% of the glycerylphosphorylcholine phosphorus and 24% of the combined glycerylphosphorylinositol and phosphorylinositol phosphorus.

Percentage of an alkali-stable acid-labile phospholipid (plasmalogen)

$$= \frac{P_X}{P_T} \times \frac{0{\cdot}8}{0{\cdot}3} \times \frac{2}{1{\cdot}6} \times 100.$$

Percentage of a phospholipid stable to mild acid and alkaline hydrolysis

$$= \frac{P_X}{P_T} \times \frac{0{\cdot}5}{0{\cdot}2} \times \frac{2}{1{\cdot}6} \times 100.$$

Micro-modification of the method

A modification with 100–200 μg. of lipid phosphorus enabled a phospholipid distribution to be obtained on small samples of tissue (50–200 mg. wet wt.). The procedure was the same as described in the macro-method except that the mild alkaline hydrolysis was performed with 0·4 N-NaOH in place of the 1 N-NaOH. For chromatography 0·5 ml. of the mild-alkaline hydrolysate (A) was used and all of the hydrolysate B (plasmalogens) and C (lipids stable to mild acid and alkaline hydrolysis). The volume of these samples was decreased for convenient application to the paper, by evaporating *in vacuo* in small glass flasks (10 ml.) drawn to a point at the bottom. Transference of the hydrolysate from the flask to the paper was facilitated by colouring it with a little bromothymol blue. This ran to the solvent front on subsequent chromatography in the phenol solvent but was readily distinguishable from the glycerylphosphorylcholine spot.

Recoveries of the lipid phosphorus hydrolysed tended to be low (80–90%). Since, however, the losses can largely be attributed to non-quantitative transference of the hydrolysates to the paper, the percentage distribution of phospholipids can be calculated with little error by expressing the recovery from the spots as a percentage of the total phosphorus recovered rather than of that hydrolysed as in the macro-method.

Materials

Ovolecithin and monophosphoinositide were prepared by previously described methods (Dawson, 1958*b*). Phosphatidylethanolamine was prepared by fractionating yeast kephalin on a silicic acid column according to Lea, Rhodes & Stoll (1955). Phosphatidylserine and diphosphoinositide were prepared from ox brain by the methods of Folch (1949*a*, *b*). Cardiolipin was donated by Dr M. G. Macfarlane of the Lister Institute and sphingomyelin by Dr J. Olley of the Torry Research Station.

Sodium-α-glycerophosphate was a preparation supplied by Boots Ltd., sodium inositol monophosphate was given by Dr J. N. Hawthorne, Birmingham, and brucine cyclic glycerol 1:2-phosphate by Dr D. Brown, Cambridge.

RESULTS

Mild alkaline hydrolysis of phospholipids. In initial experiments attempts were made to deacylate the phospholipids with volatile bases, e.g. ammonia or diethylamine. However, only very slow hydrolysis of the fatty-acyl-ester bonds was achieved. When sodium hydroxide was used as the base it was found that, provided that slight excess of alkali was present at the end of the hydrolysis, complete deacylation of the alkali-labile phospholipids as measured by appearance of acid-soluble phosphorus or by maximal loss of acyl-ester bonds occurred within 20 min. at 37°. In the present method the sodium hydroxide concentration was reduced to a point which just ensured that with normal lipid extracts there would be slight excess present at the end of the hydrolysis.

This excess was 'neutralized' with ethyl formate, which automatically protects the system against acidification beyond the neutral point, which would cause hydrolysis of the plasmalogens. The resultant sodium formate in the hydrolysate forms a large alkali spot on the chromatogram, which although not affecting the quantitative recovery of phosphorus from the spots does tend to distort two-dimensional chromatograms somewhat, by obscuring the cyclic glycerophosphate spot. However, by using Amberlite IRC 50 resin to remove the alkali (method II) this difficulty can be avoided, but in this case the non-alkali-labile lipids are partly removed by the resin and cannot be subsequently examined.

Hydrolysis of plasmalogens. It is now generally recognized that after alkaline hydrolysis the aldehyde grouping of the plasmalogens becomes extremely labile to acid hydrolysis (Feulgen & Bersin, 1939; Schmidt, Benotti, Herschman & Thannhauser, 1946; Schmidt, Ottenstein & Bessman, 1953). This observation was applied in the present method, the water-soluble products formed on acid hydrolysis being examined by paper chromatography. Trichloroacetic acid was chosen as the acid for the hydrolysis because it could be subsequently removed from the aqueous hydrolysate by ether extraction.

Two lipid fractions rich in plasmalogen were investigated in preliminary experiments. These were ram-spermatozoa phospholipids rich in choline plasmalogen (Lovern, Olley, Hartree & Mann, 1957; the present experiments indicated that some ethanolamine plasmalogen was also present) and brain phospholipids which contain ethanolamine plasmalogen and to a less extent serine plasmalogen (Klenk & Böhm, 1951). The alkali-stable phospholipids from these sources were dissolved in *iso*butanol–chloroform (1:2, v/v) and shaken vigorously with 10% trichloroacetic acid solution at 37°. Maximum liberation of water-soluble phosphorus occurred within 20 min. Paper chromatography showed that this phosphorus was in the form of the water-soluble glycerylphosphoryl derivatives of each plasmalogen base (i.e. glycerylphosphoryl-choline, -ethanolamine and -serine).

Hydrolysis of phospholipids resistant to mild acid and alkaline hydrolysis. Although the phosphorus

in this fraction is commonly used as a measure of sphingomyelin a number of workers have pointed out that the alkali- and acid-stable lipids of tissues contain other phospholipids (Brante, 1949; Mallov, McKibbin & Robb, 1953). Dawson (1954*b*) found that the alkali- and acid-stable phospholipids of brain possessed choline phosphorus molar ratios which were less than one and suggested that sphingomyelin could best be estimated by measuring the choline in the fraction or the difference between total choline and the choline released on mild alkaline and acid hydrolysis. Rennkamp (1949) showed that on the hydrolysis of sphingomyelin with dry methanolic hydrochloric acid, phosphorylcholine and sphingosylphosphorylcholine were produced. These results were recently confirmed by Dawson (1958*a*), who showed that both compounds moved on a phenol–water paper chromatogram as a single fast-running spot. These observations form the basis of the present method, in which that fraction of the phospholipids stable to mild alkaline and acid hydrolysis was hydrolysed for 4 hr. with dry methanolic hydrochloric acid. The total hydrolysate when chromatographed on paper in phenol–water yielded a main spot at about R_F 0·8 and subsidiary spots at lower R_F values. Pure sphingomyelin subjected to the same hydrolytic procedure (mild aqueous alkali and acid followed by methanolic hydrochloric acid) produced a single spot at R_F 0·8. Recently, Ansell & Spanner (1959) have used a similar method to measure the uptake of ^{32}P into brain sphingomyelin.

Paper chromatography of hydrolysates. Fig. 2 shows diagrammatically the approximate position of the phosphorus-containing spots when the three hydrolysates (A, B and C) were chromatographed unidimensionally in phenol-containing solvents. The identities of the spots were determined either by using the pure substances as markers or by hydrolysing the individual phospholipids and determining the position of their hydrolysis products on equivalent chromatograms. A preliminary ninhydrin spray also helped to locate the position of glycerylphosphoryl-ethanolamine and -serine.

In agreement with previous work (Dawson, 1954*a*) it was shown that when pure phosphoglycerides were subjected to mild alkaline hydrolysis small amounts of products other than the simple deacylated phospholipid were formed. Maruo & Benson (1959) have recently identified one of these products as cyclic glycerophosphoric acid. Under the present experimental conditions of alkaline hydrolysis lecithin forms about 6 % of this cyclic compound and monophosphoinositide 20 %: the other phosphoglycerides form very little. The hydrolysis of monophosphoinositide is more complicated than the other phosphoglycerides: although glycerylphosphorylinositol is the predominant product (57 %), the hydrolysate contains cyclic glycerophosphate (20 %), about 14 % of phosphorylinositol and a little glycerophosphate (3 %) as well as 7 % of an unidentified phosphorus-containing hydrolysis product. Thus caution is needed in interpreting the free glycerophosphate in an alkaline hydrolysate of phospholipids as being solely derived from phosphatidic acid. The breakdown of brain inositide was similarly complicated; a number of products were formed, the structure of which is being investigated at present.

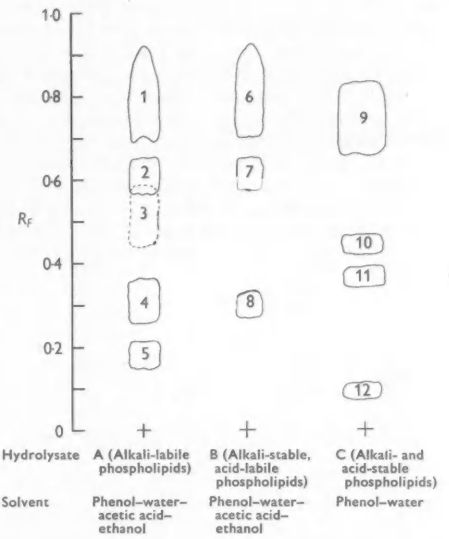

Fig. 2. Unidimensional paper chromatography of phospholipid hydrolysates, showing diagrammatically the approximate form and position of the phosphorus-containing spots. Composition of spots, with parent phospholipid in parenthesis: (1) Glycerylphosphorylcholine (lecithin); (2) glycerylphosphorylethanolamine (phosphatidylethanolamine), cyclic glycerophosphoric acid (lecithin and monophosphoinositide); (3) greenish-blue alkali spot; (4) glycerylphosphorylserine (phosphatidylserine), polyglycerolphosphoric acid (polyglycerolphospholipid), glycerophosphoric acid (phosphatidic acid; monophosphoinositide can give 3 % of glycerophosphoric acid on alkaline hydrolysis); (5) glycerylphosphorylinositol, phosphorylinositol (monophosphoinositide), hydrolysis products of brain inositide; (6) glycerylphosphorylcholine (choline plasmalogen); (7) glycerylphosphorylethanolamine (ethanolamine plasmalogen); (8) glycerylphosphorylserine (serine plasmalogen); (9) phosphorylcholine, sphingosylphosphorylcholine (sphingomyelin); (10), (11), (12) unidentified.

The identities of the minor spots 10, 11 and 12 (Fig. 2) on chromatograms of the hydrolysate of the phospholipids stable to mild alkaline and acid hydrolysis (C) are unknown. The spot 12 with very low R_F (0·09) was usually very faint and occasionally absent from the chromatogram altogether. Dr G. B. Ansell (personal communication) has observed similar spots on the paper chromatography of methanolic hydrochloric acid hydrolysates of the brain phospholipids stable to saponification by the Schmidt *et al.* (1946) procedure. Extracts of sheep-brain and -heart lipids were saponified by the same method (24 hr., 37°, N-sodium hydroxide) and chromatography of the methanolic hydrochloric acid hydrolysate confimed that similar unidentified spots were present. However, although the sphingomyelin phosphorus recovered was very similar to that in the present method of analysis the amount of phosphorus in the unidentified spots was less.

On unidimensional paper chromatography of the hydrolysate of the alkali-labile phospholipids (A), the deacylated products from phosphatidylserine, polyglycerol phospholipid (e.g. cardiolipin) and phosphatidic acid travelled more or less as a single spot (Fig. 2). If separate analysis was required, these compounds were separated by using methanol–formic acid–water as a second solvent or alternatively by using high-voltage ionophoresis at pH 3·6. Ionophoresis, as well as being more rapid, had the additional merit that with brain-lipid extracts it allowed the separation of the deacylation products of mono- and di-phosphoinositide. Fig. 3 shows a tracing of a typical two-dimensional chromatogram obtained with a mild alkaline hydrolysate (A) of sheep-brain phospholipids. Fig. 4 shows a tracing of a single-dimensional chromatogram of the alkaline hydrolysate of sheep-kidney phospholipids which had been subjected to ionophoresis at pH 3·6 in a second dimension. In Table 1 are recorded the R_F values of a number of the hydrolysis products in both the solvents phenol–water–acetic acid–ethanol and methanol–formic acid–water as well as the mobilities on ionophoresis at pH 3·6 in pyridine–acetic acid

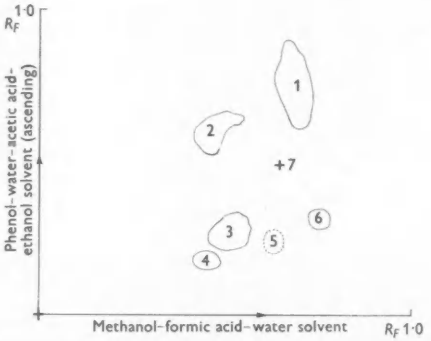

Fig. 3. Tracing of a two-dimensional paper chromatogram of the phosphorus-containing products of mild alkaline hydrolysis of 94 μg. of sheep-brain-phospholipid P. Faint spots are shown in dotted outline. Identity of spots, with parent phospholipid in parenthesis: (1) glycerylphosphorylcholine (lecithin); (2) glycerylphosphorylethanolamine (phosphatidylethanolamine); (3) glycerylphosphorylserine (phosphatidylserine); (4) glycerylphosphorylinositol + phosphorylinositol (monophosphoinositide); (5) polyglycerolphosphate (polyglycerol phospholipid); (6) glycerophosphoric acid (phosphatidic acid?); (7) approximate position of cyclic glycerophosphate (in this chromatogram the alkaline hydrolysate was prepared by method I and the alkali-metal spot obscures the spot).

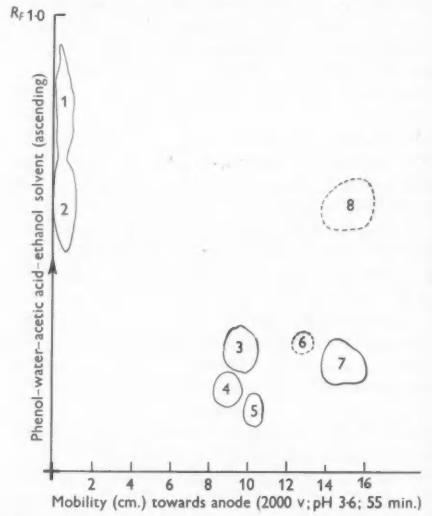

Fig. 4. Tracing of a unidimensional paper chromatogram of the phosphorus-containing products of mild alkaline hydrolysis 108 μg. of sheep-kidney-phospholipid P. The products have been separated in a second dimension with ionophoresis at pH 3·6. Faint spots are shown in dotted outline. Identity of spots, with parent phospholipid in parenthesis: (1) glycerylphosphorylcholine—this spot and spot 2 can be completely separated in descending chromatography (lecithin); (2) glycerylphosphorylethanolamine (phosphatidylethanolamine); (3) glycerylphosphorylserine (phosphatidylserine); (4) glycerylphosphorylinositol (monophosphoinositide); (5) phosphorylinositol (monophosphoinositide); (6) glycerophosphoric acid (phosphatidic acid?); (7) polyglycerolphosphoric acid (polyglycerolphospholipid); (8) cyclic glycerophosphoric acid (lecithin and monophosphoinositide).

Table 1. *Approximate R_F values and ionophoretic mobilities of the phosphorus-containing hydrolysis products produced on the mild alkaline hydrolysis of phospholipids*

Hydrolysis product	Parent phospholipid	R_F: In phenol–water–acetic acid–ethanol	R_F: In methanol–formic acid–water	Mobility at pH 3·6 relative to inorganic P
Glycerylphosphorylcholine	Lecithin	0·77	0·67	0
Glycerylphosphorylethanolamine	Phosphatidylethanolamine	0·63	0·47	0
Glycerylphosphorylserine	Phosphatidylserine	0·30	0·50	0·61
Glycerylphosphorylinositol	Monophosphoinositide	0·20	0·45	0·57
Phosphorylinositol	Monophosphoinositide	0·20	0·45	0·64
Glycerophosphoric acid	Phosphatidic acid	0·33	0·74	0·82
Polyglycerol phosphate	Polyglycerol phospholipid	0·28	0·60	0·96
Hydrolysis products of brain inositide	Brain inositide	0·12	0·66	1·0 and 1·16
Cyclic glycerophosphoric acid	Lecithin / Monophosphoinositide	0·50	0·66	1·0
Inorganic P		0·25	0·85	1·0

Table 2. *Distribution of individual phospholipids in tissues of the sheep*

Tissues were obtained from a ewe (50 kg.) as soon as possible after death (10–40 min.) and frozen in solid CO_2 until used. Results express the phosphorus of the phospholipid as a percentage of the total lipid phosphorus.

	Brain (whole)	Kidney	Liver	Skeletal muscle	Lung	Heart muscle
Total phospholipid (mg. of P/g. of wet tissue)	2·20	1·22	1·94	0·55	1·02	0·91
Phosphatidylcholine (lecithin)	38·6	36·4	55·2	39·9	48·5	27·5
Phosphatidylethanolamine	17·5	20·0	26·5	14·7	14·0	19·1
Phosphatidylserine	16·0	8·2	4·4	3·8	9·9	3·5
Monophosphoinositide	2·2	4·2	6·1	2·6	2·6	3·8
Polyglycerolphospholipid	1·1	6·7	3·2	7·0	0·8	14·5*
'Phosphatidic acid'	1·0	Trace	Trace	—	0·5	—
Choline plasmalogen	—	1·1	—	2·3	0·8	11·4
Ethanolamine plasmalogen	11·0	5·3	0·2	6·6	6·2	6·7
Serine plasmalogen	0·4	Trace	—	—	—	—
Sphingomyelin	9·4	13·2	4·2	6·4	12·4	6·6
Unidentified mild alkali- and acid-stable phospholipid(s)	5·6	3·0	0·5	3·4	1·9	7·9
Recovery (% of total lipid P analysed)	102·8	98·1	100·3	86·7	97·6	101·0

* Cardiolipin.

buffer. It must be emphasized that these R_F values and mobilities can never be regarded as absolute, and can vary slightly from day to day with the length of the chromatographic run, temperature, etc.; nevertheless, the relative positions of the spots remain constant.

Examination of the phospholipids present in sheep tissues. In Table 2 results are presented of the analysis of six tissues of the sheep by the present method. The estimations were performed in duplicate runs showing close agreement. It can be seen that very good recoveries of the lipid phosphorus used for analysis were obtained from the chromatograms, apart from skeletal muscle, where the recovery was 13% low.

Few previous estimations have been made of the concentrations of individual phospholipids present in sheep tissues. With the alkali-labile phospholipids the distribution of lecithin, phosphatidylethanolamine and phosphatidylserine (Table 2) is closely similar to that found in rat tissues (Dawson, 1957). Although the percentage of phosphorus present in polyglycerol phospholipid was highest in heart (cardiolipin) it is perhaps surprising that there was also an appreciable percentage in both the kidney and muscle lipids and to a lesser extent in liver lipids. McKibbin & Taylor (1952) isolated a polyglycerol phospholipid from horse liver and Dawson (1958*c*) has recently isolated small amounts of a similar compound from rat and guinea-pig liver. A very faint glycerophosphate spot which may correspond to true phosphatidic acid was seen on certain chromatograms. Glycerophosphate is known to be the major product

formed by the mild alkaline hydrolysis of phosphatidic acid (Dawson, 1954*a*; Hübscher & Clark, 1959), but small amounts can also be derived from monophosphoinositide hydrolysis. Hokin & Hokin (1958) recently demonstrated by an isotopic technique the presence of traces of phosphatidic acid in mouse tissues whereas Hübscher & Clark (1959) have isolated about 0·5–1 % of phosphatidic acid from the phospholipids of ox liver.

The analysis of the individual tissue plasmalogens generally agrees with the small amount of evidence at present available on the distribution of tissue plasmalogens obtained by isolation experiments. Thus Feulgen & Bersin (1939) first isolated ethanolamine plasmalogen from muscle and Klenk & Böhm (1951) found that appreciable amounts were also present in brain phospholipids. The present results (Table 2) indicate a similar trend in the tissues of the sheep but also show that ethanolamine plasmalogen is the predominant plasmalogen in the kidneys and lungs. The small amount of plasmalogen in the liver consisted almost entirely of ethanolamine plasmalogen, whereas in heart muscle although choline plasmalogen was predominant, appreciable quantities of ethanolamine plasmalogen were also present. Klenk & Gehrmann (1953) and Rapport & Alonzo (1955) have found that the lecithin fraction of ox heart is rich in choline plasmalogen.

Of the phospholipids stable to mild alkaline and acid hydrolysis, brain, lung and kidney lipids appeared to be the richest sources of sphingomyelin whereas brain and heart-muscle lipids possessed appreciable amounts of phospholipid(s) resistant to alkali and acid hydrolysis, which was not sphingomeylin. In heart muscle this unidentified phospholipid(s) exceeded the amount of sphingomyelin, whereas in liver very little was present.

DISCUSSION

The present method of analysing the phospholipid fraction of a tissue has certain advantages over conventional methods of analysis. The method requires only small amounts of tissue and as only one type of estimation is required (phosphorus) it is rapidly carried through. Further, it enables estimates to be made of phospholipids which have hitherto been detected only by isolation experiments, e.g. cardiolipin, and it resolves the serine, ethanolamine and choline phosphoglycerides into their individual plasmalogen and di-acylated components. No resolution of lecithin and phosphatidylethanolamine from their respective lyso compounds is possible with the present method. In fresh tissues this may not be a serious disadvantage as the amount of lysolecithin appears to be small compared with that of lecithin itself (Marinetti, Witter & Stotz, 1957); in autolysed tissues it may be a more important factor.

The question arises of the identity of the phospholipids which occur in the fraction stable to mild acid and alkaline hydrolysis and which are not sphingomyelin. Carter, Smith & Jones (1958) have recently isolated a new alkali- and acid-stable phospholipid from egg yolk which they identified as the phosphorylethanolamine derivative of batyl alcohol. Evidence was obtained that a similar compound was present in mammalian tissues and it is possible that this would partially account for the present unidentified phospholipid(s).

Comparison of the values in Table 2 shows that the amount of the unidentified phospholipid(s) in a tissue appears to be approximately directly proportional to the total plasmalogen P present in the tissue. This raises the question whether a metabolic relationship exists between the two fractions or if the unidentified lipid represents a type of plasmalogen complex which is not susceptible to mild alkaline and acid hydrolysis.

Note added in proof. Additional evidence has now been obtained that part at least of the unidentified phosphorus in the alkali- and acid-stable fraction is related to the plasmalogens. If the 10 % trichloroacetic acid solution used to hydrolyse the lysoplasmalogens is made 5 mM with respect to mercuric chloride about 20–30 % more water-soluble phosphorus is liberated on shaking at 37° for 30 min. [cf. Schmidt *et al.* (1959) *Amer. J. Dis. Child.* **97**, 691]. This extra phosphorus is derived from the unidentified phosphorus and is released as glycerylphosphoryl-choline or -ethanolamine according to the plasmalogens already present. Work is continuing to determine whether this non-acid-labile plasmalogen represents some different type or combined form of plasmalogen or if it is an artifact of the acid hydrolysis.

SUMMARY

1. A method is presented for the rapid quantitative determination of the individual phospholipids in a small sample of biological material.

2. The products of selective hydrolytic degradation are separated by paper chromatography and their phosphorus contents measured. From these, the nature and amounts of the phospholipids originally present are inferred, appropriate corrections being based upon the hydrolysis of reference compounds.

3. By this method, the distribution of lecithin, phosphatidylethanolamine, phosphatidylserine, monophosphoinositide, polyglycerol phospholipid (cardiolipin), 'phosphatidic acid', choline plasmalogen, ethanolamine plasmalogen, serine plasmalogen and sphingomyelin has been measured in six tissues of the sheep.

I wish to acknowledge the expert technical assistance of Miss N. Hemington.

REFERENCES

Allen, R. J. L. (1940). *Biochem. J.* **34**, 858.
Ansell, G. B. & Spanner, S. (1959). *Biochem. J.* **73**, 3P.
Barron, E. J. & Hanahan, D. J. (1958). *J. biol. Chem.* **231**, 493.
Borgström, B. (1952). *Acta physiol. scand.* **25**, 101.
Brante, G. (1949). *Acta physiol. scand.* **18**, Suppl. 63.
Carter, H. E., Smith, D. B. & Jones, D. N. (1958). *J. biol. Chem.* **232**, 681.
Dawson, R. M. C. (1954*a*). *Biochim. biophys. Acta*, **14**, 374.
Dawson, R. M. C. (1954*b*). *Biochem. J.* **56**, 621.
Dawson, R. M. C. (1957). *Biol. Rev.* **32**, 188.
Dawson, R. M. C. (1958*a*). *Biochem. J.* **68**, 357.
Dawson, R. M. C. (1958*b*). *Biochem. J.* **70**, 559.
Dawson, R. M. C. (1958*c*). *Biochem. J.* **68**, 352.
Feulgen, R. & Bersin, T. (1939). *Hoppe-Seyl. Z.* **260**, 217.
Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
Folch, J. (1949*a*). *J. biol. Chem.* **174**, 439.
Folch, J. (1949*b*). *J. biol. Chem.* **177**, 505.
Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957). *J. biol. Chem.* **226**, 497.
Hanes, C. S. & Isherwood, F. A. (1949). *Nature, Lond.*, **164**, 1107.
Hokin, L. E. & Hokin, M. R. (1958). *J. biol. Chem.* **233**, 800.
Hübscher, G. & Clark, B. (1959). *Biochem. J.* **72**, 7P.
Klenk, E. & Böhm, P. (1951). *Hoppe-Seyl. Z.* **288**, 98.
Klenk, E. & Gehrmann, G. (1953). *Hoppe-Seyl. Z.* **292**, 110.
Lea, C. H., Rhodes, D. N. & Stoll, R. D. (1955). *Biochem. J.* **60**, 353.
Lovern, J. A., Olley, J., Hartree, E. F. & Mann, T. (1957). *Biochem. J.* **67**, 630.
McKibbin, J. M. & Taylor, W. E. (1952). *J. biol. Chem.* **196**, 427.
Mallov, S., McKibbin, J. M. & Robb, J. S. (1953). *J. biol. Chem.* **201**, 825.
Marinetti, G. V., Witter, R. F. & Stotz, E. (1957). *J. biol. Chem.* **226**, 475.
Maruo, B. & Benson, A. A. (1959). *J. biol. Chem.* **234**, 254.
Rapport, M. M. & Alonzo, N. (1955). *J. biol. Chem.* **217**, 199.
Rennkamp, F. (1949). *Hoppe-Seyl. Z.* **284**, 215.
Ryle, A. P., Sanger, F., Smith, L. F. & Kitai, R. (1955). *Biochem. J.* **60**, 541.
Schmidt, G., Benotti, J., Herschman, B. & Thannhauser, S. J. (1946). *J. biol. Chem.* **166**, 505.
Schmidt, G., Ottenstein, B. & Bessman, M. J. (1953). *Fed. Proc.* **12**, 265.

Biochem. J. (1960) **75**, 53

Studies in Flavinogenesis

6. THE ROLE OF THREONINE IN RIBOFLAVIN BIOSYNTHESIS IN *EREMOTHECIUM ASHBYII**

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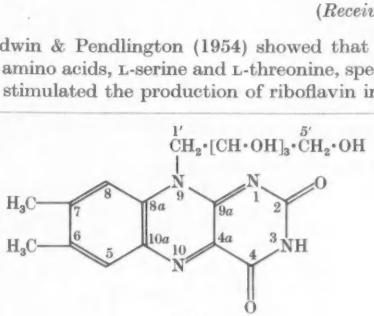
Goodwin & Pendlington (1954) showed that only two amino acids, L-serine and L-threonine, specifically stimulated the production of riboflavin in the mould *Eremothecium ashbyii*. The mechanism of stimulation by L-serine was elucidated by Goodwin & Jones (1956), using [^{14}C]serine. It had already been shown by Plaut (1954) that C-4*a* and C-9*a* of riboflavin (I) in *Ashbya gossypii* arise from glycine and that C-2 is derived from formate, and Goodwin & Jones demonstrated that C-1 and C-2 of serine were found in C-4*a* and 9*a* of riboflavin and C-3 of serine in C-2 of riboflavin. Thus serine was incorporated into the riboflavin molecule after being split into a C_1 unit (formate) and glycine.



(I)

The mechanism of threonine incorporation has now been studied with L-[^{14}C]threonine. Part of this work has already been briefly reported (Goodwin & Horton, 1959).

EXPERIMENTAL

Organism. Our normal strain of *Eremothecium ashbyii* (Brown, Goodwin & Jones, 1958) was used throughout the investigation.

Medium. The medium used was the basal medium described by Goodwin & Pendlington (1954) with bacteriological peptone added to a concentration of 0·04%. After adjusting to pH 5·8, the medium was autoclaved at 15 lb./in.^{2} for 15 min.

* Part 5. Goodwin & McEvoy (1959).

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Cultural conditions. The organism was grown at 28° in continual-shake culture in 250 ml. Erlenmeyer flasks, each containing 50 ml. of medium. The flasks were inoculated with 2 ml. of a 48 hr. culture of *E. ashbyii* (Goodwin & Pendlington, 1954).

Isolation of riboflavin. The cells from the culture flasks were combined and filtered. The residual yellow mycelium was boiled with water for about 1 min. and again filtered; the residue was white. The combined filtrates were evaporated to small volume under reduced pressure and absolute ethanol (five times the volume of the concentrate) was added. The resulting precipitate, containing much protein and a trace of flavinadenine dinucleotide, was filtered off and the filtrate evaporated to dryness *in vacuo*. The residue was dissolved in a minimal volume of water and the pH adjusted when necessary with dilute hydrochloric acid to between 5·0 and 5·5. A freshly prepared saturated solution of sodium dithionite was added dropwise until all the riboflavin was reduced, as indicated by the sudden disappearance of the characteristic greenish fluorescence in u.v. light. The air in the reaction flask was immediately replaced by nitrogen and the flask stoppered and kept overnight at 4°. Next day the precipitated leucoriboflavin was centrifuged off and washed three times with dilute sodium dithionite solution and then twice with ice-cold water. The leucoriboflavin was dissolved in warm water and oxidized by blowing air through the solution, which was subsequently filtered. The filtrate was then decreased in volume by vacuum distillation, until riboflavin just began to crystallize out. The solution was immediately filtered and left at 0–4° to crystallize. The crystals were centrifuged and washed with small volumes of ice-cold water. The purity of the riboflavin was checked by spectroscopic measurement at 266 and 445 mμ (Adamson, 1948), and by paper chromatography in butanol–acetic acid–water (4:1:5, by vol.) once in each phase (i.e. one chromatogram was run successively in each phase, the direction of solvent flow being the same in each case).

Degradation of riboflavin

Conversion into lumiflavin and lumichrome. Riboflavin (32·5 mg.) was dissolved in 200 ml. of 0·5 N-sodium hydroxide and the solution placed in a crystallizing dish (16 cm. diam.) which gave a depth of liquid of 1 cm. The dish was placed in a copper container through which cold water was continuously circulated so that the temperature of the riboflavin solution never rose above 15°. The solution was agitated with a magnetic stirrer and irradiated for 8 hr. with a glass-envelope mercury-arc lamp (Phillips 120 w at a distance of 15 cm.). This source of u.v. radiation gave better results than an unfiltered mercury arc in a quartz envelope (Hanovia) previously used in this Laboratory (Goodwin & Jones, 1956). The solution was then adjusted to pH 5·0 with acetic acid and the resulting lumiflavin and lumichrome were extracted repeatedly with chloroform. The combined chloroform extracts were evaporated to small volume and chromatographed on paper with the butanol–acetic acid–water system (organic phase). This separated lumiflavin and lumichrome well. The lumiflavin was eluted with warm water and crystallized

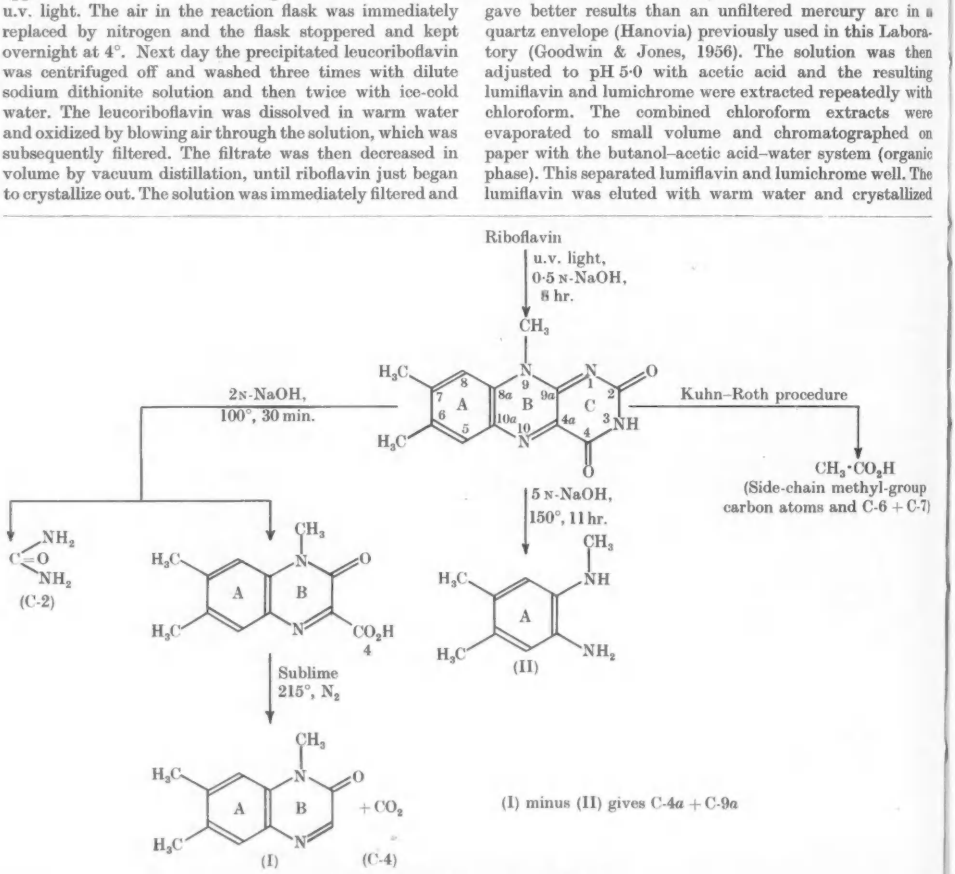

Fig. 1. Degradation of riboflavin.

from methanol. Its purity was checked spectrophotometrically by the use of the data of McNutt (1956), and by rechromatographing the sample in the aqueous phase of the butanol–acetic acid–water system, when it was found that its specific activity remained constant.

Degradation to N-*methyl*-1:2-*diamino*-4:5-*dimethylbenzene*. Lumiflavin (30 mg.) was dissolved in 5N-sodium hydroxide (16 ml.) and the solution heated at 150° for 10 hr. in a stainless-steel bomb. The cooled reaction mixture was extracted five times with 50 ml. portions of diethyl ether. The combined extracts were evaporated to dryness *in vacuo* and the residue was dissolved in 50 ml. of dry ether. The solution was allowed to stand over pellets of potassium hydroxide for 4–5 hr.; the ether was then removed *in vacuo* and the residue dried overnight in a vacuum desiccator (over sulphuric acid). The dry residue was dissolved in dry peroxide-free ether and transferred to a 15 ml. centrifuge tube. The dihydrochloride of *N*-methyl-1:2-diamino-4:5-dimethylbenzene was precipitated by passing dry hydrogen chloride gas through the solution. The hydrochloride was centrifuged down, washed with dry ether and stored in a desiccator before determination of its radioactivity.

Isolation of C-6 *and* C-7 *and the* C-*methyl groups*. Lumichrome was oxidized to acetic acid by the conventional procedure of Kuhn & Roth (1933). The acetic acid was collected by steam distillation and converted into its sodium salt, which was counted. By this means C-6 + C-7 + C-methyl groups were isolated.

Isolation of C-2 *and* C-4. Lumiflavin (30 mg.) was heated with 2N-sodium hydroxide at 100° for 30 min. and the resulting urea (C-2) was isolated as dixanthyl urea (Goodwin & Jones, 1956). The keto acid formed at the same time was isolated and sublimed at 215° in an atmosphere of nitrogen; C-4 was liberated as carbon dioxide, which was collected in barium hydroxide (Goodwin & Jones, 1956).

Isolation of C-4*a* + C-9*a*. The lactam obtained after decarboxylating the keto acid (see previous paragraph) was isolated as previously described (Goodwin & Jones, 1956). The activity in C-4*a* + C-9*a* is obtained by subtracting the activity of *N*-methyl-1:2-diamino-4:5-dimethylbenzene from that of the lactam.

An outline of the degradations used is given in Fig. 1.

Preparation of crude homogenates. Cells of *E. ashbyii* were harvested after incubation for 48 hr., washed twice with 0·15M-phosphate buffer, pH 7·6, suspended in the minimum volume of the same buffer and disintegrated at 0° in a small Waring Blendor.

Determination of threonine aldolase. The microdiffusion method of Karasek & Greenberg (1957) was employed, conventional Conway units being used.

Labelled materials. L-[^{14}C]Threonine was purchased from A.E.R.E., Harwell, Bucks.

Measurements of radioactivity. All activities were measured on a windowless gas-flow counter (Nuclear Measurements Corp. Inc., Chicago, Ill., U.S.A.) as previously described (Goodwin, 1958).

RESULTS

Incorporation of L-[^{14}C]*threonine into riboflavin by* Eremothecium ashbyii

L-[^{14}C]Threonine is actively incorporated into riboflavin under our experimental conditions, in which the labelled material is added to the cultures 48 hr. after inoculation and the riboflavin is extracted after a further 48 hr. Percentage incorporations up to 4·5 have been obtained and these figures are similar to those obtained with generally labelled L-[^{14}C]serine, where the maximum uptake recorded was in the region of 7 % (Goodwin & Jones, 1956). Degradation of the resulting labelled riboflavin as described in the Experimental section indicated that the activity was entirely confined to C-4*a* and C-9*a* (Table 1). These are the carbon atoms which normally arise from glycine (Plaut, 1954) and thus threonine must be metabolized to glycine before incorporation into riboflavin.

Braunshtein & Vilenkina (1949) reported the presence of an enzyme, threonine aldolase, which splits threonine into glycine and acetaldehyde, and this has been considerably purified from sheep liver by Karasek & Greenberg (1957); it is also present in much lower activity in yeast. The existence of this enzyme in *E. ashbyii* would account for the labelling pattern observed in riboflavin synthesized in the presence of L-[^{14}C]-threonine (Table 1). Its presence has now been demonstrated in crude homogenates of *E. ashbyii* with the technique of Karasek & Greenberg (1957). Samples of homogenates were placed in the outer compartments of Conway units and a solution of semicarbazide was placed in the inner compartments. The production of semicarbazone was determined by measuring $E_{224\,m\mu}$ of the solution in the centre well. Table 2 shows that on incubating L-threonine with homogenates of *E. ashbyii*, acetaldehyde is produced in excess of that formed endogenously. The percentage conversion is, however, low (0·06 %). As the endogenous acetaldehyde formation was always relatively high in

Table 1. *Incorporation of generally labelled* L-[^{14}C]*threonine into riboflavin by* Eremothecium ashbyii

Labelled threonine was added to 48 hr. cultures and riboflavin was isolated 48 hr. later.

Compound isolated	Specific activity (counts/min./μmole)
Riboflavin	4100
Lumiflavin	4300
Acetic acid (C-6, C-7 plus adjacent methyl groups)	0
Dixanthylurea (C-2)	0
Carbon dioxide (C-4)	0
N-Methyl-1:2-diamino-4:5-dimethylbenzene (aromatic ring + C-1 + side-chain methyl groups)	0
Lactam (carbon atoms in *N*-methyl-1:2-diamino-4:5-dimethylbenzene plus C-4*a* + C-9*a*)	4100

Table 2. *Formation of acetaldehyde from threonine by homogenates of* Eremothecium ashbyii

Homogenates were produced as described in the Experimental section; L-threonine (125 μmoles) was in the outer compartment of the Conway unit; temp. 27°; time 2 hr.

Material in outer compartment	Acetaldehyde produced (μmole)	
Threonine alone	0·0349	0·0291
Boiled homogenate	0·0622	0·0627
Homogenate alone	0·0956	0·1020
Homogenate plus threonine	0·2045	0·2115
	0·2159	0·2203

Table 3. *Formation of* [^{14}C]*acetaldehyde from generally labelled* L-[^{14}C]*threonine by homogenates of* Eremothecium ashbyii

Experimental conditions were as described in Table 2, but with incubation time 24 hr.

	Counts/min.
Activity of L-[^{14}C]threonine added to each unit	$0{\cdot}5 \times 10^6$
Activity of acetaldehyde recovered from centre well of each unit	850, 590, 720, 740
Activity in centre well of unit with L-[^{14}C]threonine but no homogenate present in outer compartment	20, 38

spite of various attempts to decrease it, the reality of the enzymic reaction was confirmed by using L-[^{14}C]threonine as substrate. Table 3 demonstrates that significant activity is found in the centre well when the labelled L-threonine is incubated with homogenates of *E. ashbyii*. The amount of radioactivity recovered in the centre well represents a conversion of 0·14 %, which is very similar to that found in the series of experiments quoted in Table 2. In order finally to demonstrate that acetaldehyde was being formed, unlabelled acetaldehyde was added to the combined contents of the centre wells of the units used in the radioactive experiment, and the acetaldehyde semicarbazone was allowed to crystallize. The crystalline material was found to be radioactive and it retained its activity after further recrystallization.

DISCUSSION

The results obtained in this investigation make it clear that C-1 and C-2 of threonine are incorporated by *Eremothecium ashbyii* into C-4*a* and C-9*a* of riboflavin after cleavage of the threonine to glycine and acetaldehyde by threonine aldolase. As serine and threonine are the only specifically flavinogenic amino acids under our conditions (Goodwin & Pendlington, 1954), and as previous isotope work has indicated that serine provides both formate and glycine for riboflavin synthesis (Goodwin & Jones, 1956), it follows that glycine must be the limiting factor in the biosynthetic pathway in *E. ashbyii*. As glycine itself fails to stimulate flavinogenesis (Goodwin & Pendlington, 1954), the results with serine and threonine suggest that they are stimulatory for one of two reasons: (*a*) serine and threonine aldolases liberate an activated form of glycine and the organism either lacks the ability to activate glycine or activates it so slowly compared with the rate of formation of endogenous 'active glycine' that no stimulation of flavinogenesis can be observed; (*b*) the cell wall of *E. ashbyii* is permeable to serine and threonine but not to glycine. The first possibility is probably correct, but as tracer amounts of [^{14}C]glycine are incorporated into riboflavin in the closely related *Ashbya gossypii* (Plaut, 1954), *E. ashbyii* can probably activate glycine, but only very slowly. The observation that *E. ashbyii* does not use glycine for growth (Dulaney & Grutter, 1950) cannot be taken as indicating that it is not metabolized to a small extent; indeed, neither threonine nor serine stimulates growth in *E. ashbyii* (Goodwin & Pendlington, 1954).

The fact that up to 4 % of added [^{14}C]threonine is incorporated into riboflavin in intact cells of *E. ashbyii* compared with the conversion of only 0·14 % of the threonine into glycine and acetaldehyde by crude homogenates in 24 hr. suggests that threonine aldolase is quickly inactivated on breaking up the cells. This may be the explanation of the low activity of threonine aldolase observed by Karasek & Greenberg (1957) in yeast extracts compared with liver extracts.

The present experiments indicate that the failure of homoserine to stimulate flavinogenesis in *E. ashbyii* (Brown, 1956) is almost certainly due to the fact that an enzyme does not exist which will liberate 'active glycine' from homoserine. The observations of Braunshtein & Vilenkina (1949) and Karasek & Greenberg (1957), that threonine aldolase also acts effectively on L-allothreonine, indicate that L-allothreonine should also be strongly flavinogenic in *E. ashbyii*; Pendlington (1955) reported that this is indeed the case.

The failure of any activity to appear in the aromatic ring of riboflavin formed in the presence of generally-labelled threonine demonstrates that free acetaldehyde is not an intermediate in the formation of the aromatic ring, although we have found that homogenates of *E. ashbyii* rapidly decarboxylate pyruvate with the production of acetaldehyde and Goodwin & Treble (1958) have shown that the aromatic ring probably arises from pyruvate via a C_4 compound such as acetoin. The present results indicate that a C_4 compound of this type cannot be formed by the condensation of one molecule of pyruvate and one molecule of

acetaldehyde with the liberation of carbon dioxide, thus:

$$CH_3 \cdot CO \cdot CO_2H + CHO \cdot CH_3 \xrightarrow{-CO_2} CH_3 \cdot CO \cdot CH(OH) \cdot CH_3.$$

SUMMARY

1. *Eremothecium ashbyii* incorporates generally-labelled L-[^{14}C]threonine into riboflavin to the extent of 4%.

2. C-1 and C-2 of threonine are incorporated as glycine into C-4*a* + C-9*a* of riboflavin. C-3 and C-4 of threonine are not incorporated into riboflavin.

3. The enzyme threonine aldolase, splitting threonine into glycine (C-1 + C-2) and acetaldehyde (C-3 + C-4), is present in *E. ashbyii*.

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REFERENCES

Adamson, D. C. M. (1948). *Analyst*, **73**, 442.
Braunshtein, A. E. & Vilenkina, G. Y. (1949). *C.R. Acad. Sci. U.R.S.S.* **66**, 1343.
Brown, E. G. (1956). Ph.D. Thesis: University of Liverpool.
Brown, E. G., Goodwin, T. W. & Jones, O. T. G. (1958). *Biochem. J.* **68**, 40.
Dulaney, E. L. & Grutter, F. H. (1950). *Mycologia*, **42**, 717.
Goodwin, T. W. (1958). *Biochem. J.* **70**, 612.
Goodwin, T. W. & Horton, A. A. (1959). *Biochem. J.* **73**, 13P.
Goodwin, T. W. & Jones, O. T. G. (1956). *Biochem. J.* **64**, 9.
Goodwin, T. W. & McEvoy, D. (1959). *Biochem. J.* **71**, 742.
Goodwin, T. W. & Pendlington, S. (1954). *Biochem. J.* **57**, 631.
Goodwin, T. W. & Treble, D. H. (1958). *Biochem. J.* **70**, 14P.
Karasek, M. A. & Greenberg, D. M. (1957). *J. biol. Chem.* **227**, 191.
Kuhn, R. & Roth, M. (1933). *Ber. dtsch. chem. Ges.* **66**, 1274.
McNutt, W. S. (1956). *J. biol. Chem.* **219**, 365.
Pendlington, S. (1955). Ph.D. Thesis: University of Liverpool.
Plaut, G. W. E. (1954). *J. biol. Chem.* **208**, 513.

Biochem. J. (1960) **75**, 57

The Lactic Dehydrogenase of *Propionibacterium pentosaceum*

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The occurrence in *Propionibacterium pentosaceum* of enzymes which are able to catalyse an oxidation of lactate in the presence of fumarate has been demonstrated by the work of Barker & Lipmann (1944). The evidence presented by these authors suggested the participation in the system of a type of lactic dehydrogenase different from that occurring in animal tissues.

They worked exclusively with dried cell preparations and their attempts to extract active fractions from such cells failed. A crude preparation, which carried out a fast lactate–fumarate dismutation, was obtained by Lara (1954) by grinding frozen cells of *P. pentosaceum* with alumina, essentially as described by McIlwain (1948).

The present paper describes the methods of preparation and some properties of a fraction capable of catalysing the oxidation of lactate in the presence of several redox-dyes, and also some inhibition studies.

MATERIALS AND METHODS

Organism and media. *P. pentosaceum* strain E. 2·1, obtained from Professor C. B. van Niel's collection, was cultured and harvested essentially as described by Barker & Lipmann (1944); tryptone, however, was omitted from the medium.

Methods. All reagent solutions were prepared with twice-distilled water, and the enzyme dilutions were made with 0·5% (w/v) bovine plasma-albumin solution (Armour). All operations with the enzyme were done in a cold room maintained at 0–4°, and the centrifugings were performed in a Servall centrifuge, model SS-1, at 25 000 *g* for 15 min. unless otherwise specified. Oxygen consumption was determined in a Warburg apparatus of conventional type, and the extinction determinations were made in 3 ml. cuvettes on a Beckman spectrophotometer model DU. Protein was determined by the method of Gornall, Bardawill & David (1949), with a coefficient of 0·095 (extinction at 540 mμ, 1 cm. light path, given by a solution of 1 mg. of protein/3 ml. of reaction mixture in the presence of 1·5 ml. of biuret reagent), and pyruvate was estimated by the

method of Friedemann & Haugen (1943), simplified by omitting the extraction of the phenylhydrazone.

Perchloric acid extracts were obtained from the purified enzyme preparations as follows: 60 % (w/v) perchloric acid was added to the purified enzyme to obtain 0·6 N final concentration. The precipitated proteins were separated by centrifuging and the supernatant was neutralized with KOH; the precipitated perchlorate was separated by filtration. One unit of perchloric acid extract is defined as the amount contained in the neutralized supernatant obtained from a solution containing initially one indophenol unit of lactic dehydrogenase.

The preparation used in the inhibition studies had a specific activity of 4·7 indophenol units/mg. of protein. The inhibitory action was estimated usually by the effect upon activity while the enzyme was being determined by the indophenol assay, in which case all the assay components plus the inhibitor were added to the cuvettes and the reaction was started by enzyme addition. Dicoumarol was dissolved in 0·01 N-KOH, and in this case the results obtained were compared with blank enzyme assays in which the same amount of 0·01 N-KOH was added.

Chemicals. Adenosine monophosphate (AMP), adenosine triphosphate (ATP), diphosphopyridine nucleotide (DPN), reduced diphosphopyridine nucleotide (DPNH), triphosphopyridine nucleotide (TPN), flavin mononucleotide (FMN), flavinadenine dinucleotide (FAD), cytochrome *c* and 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer were obtained from the Sigma Chemical Co. (St Louis, Mo., U.S.A.). Protamine sulphate was obtained from Eli Lilly and Co., Indianopolis, Ind., U.S.A., D-(+)-lactic acid and L-(−)-lactic acid were obtained from the Delta Chemical Works, New York, U.S.A. and disodium dihydrogen ethylenediaminetetra-acetate (EDTA) was obtained from City Chemical Corp., New York, U.S.A. Phenazine methosulphate was synthesized according to Singer & Kearney (1957), 1:2-dihydroxybenzene-3:5-disulphonate was the kind gift of Dr V. Massey. Sodium pyruvate and thyroxine were obtained from F. Hoffmann–La Roche and Co., Basel, Switzerland; sodium 5-ethyl-5-*iso*amylbarbiturate (Amytal) was from Eli Lilly and Co. of Brazil, São Paulo, Brazil; sodium 5-methyl-5-(1-methylbutyl)barbiturate (Nembutal) from Abbot Laboratório do Brasil Ltda., São Paulo, Brazil; pentachlorophenol from Eastmann Kodak Co., Rochester, N.Y., U.S.A.; 3-chloro-7-methoxy-9-(1-methyl-4-diethylaminobutylamino)-acridine dihydrochloride (quinacrine) and 7-chloro-4-(4-diethylamino-1-methylbutylamino)-quinoline diphosphate (chloroquine) were from the Winthrop Products Inc., N.Y., U.S.A.; quinine monohydrochloride was from Pelletier Delondre et Sevaillant, Armet de Lise et Cie., Paris, France. Dicoumarol [3:3′-methylenebis-(4-hydroxycoumarine] and *o*-iodosobenzoic acid were gifts from Dr Isaias Raw. Vitamin K_1 (aqueous emulsion) and menadione (2-methyl-1:4-naphthaquinone) diphosphate (aqueous solution) were used in the form of pharmaceutical products Kanakon and Synkavit respectively, and were provided by Products Roche Químicos e Farmacêuticos S.A., Rio de Janeiro, Brazil.

Enzyme assays

In the course of the purification and study of the properties of the enzyme several assay systems were used, involving spectrophotometric and manometric methods which are described below.

Indophenol assay. In a 3 ml. cuvette are placed 60 μmoles of potassium phosphate buffer, pH 7·3, 50 μmoles of racemic sodium lactate, 0·1 μmole of 2:6-dichlorophenolindophenol, 5 μmoles of disodium dihydrogen EDTA and water plus enzyme to give a final volume of 3 ml. The reaction is initiated by enzyme addition and its course estimated by measurements of extinction at 600 mμ with water as a blank. Readings are taken every 30 sec. during 2 min. In the absence of lactate the extinction changes are negligible under these conditions. The amount of enzyme used is such that the range of extinction change/min. is 0·020–0·100. Under these conditions the reaction obeys zero-order kinetics until 75 % of the dye is reduced, and the velocity of dye reduction is strictly proportional to the amount of enzyme added.

One indophenol unit is defined as the amount of enzyme which catalyses the reduction of 1 μequiv. of dye/min., at room temperature (25–27°). Specific activity was measured in indophenol units/mg. of protein. The molar-extinction coefficient for 2:6-dichlorophenolindophenol used was that reported by Basford & Huennekens (1955).

Quinone assay. The conditions are the same as for the indophenol assay, 1 μmole of 1:2-naphthaquinone-4-sulphonate being substituted for 2:6-dichlorophenolindophenol and the extinction measurements are made at 305 mμ.

One quinone unit is the amount of enzyme capable of reducing 1 μequiv. of 1:2-naphthaquinone-4-sulphonate/min. The extinction coefficient for this substance was taken as $3{\cdot}7 \times 10^6$ cm.^{2} mole^{-1}.

Methylene blue and phenazine methosulphate assays. In the main compartment of a Warburg vessel are placed 100 μmoles of potassium phosphate buffer, pH 7·3, water and the enzyme solution. The side arm contains either 3 μmoles of methylene blue or 5·4 μmoles of phenazine methosulphate and 40 μmoles of racemic sodium lactate; total volume, 2 ml. After equilibration at 30° for 5 min. the reaction is started by tipping the contents of the side arm into the main compartment. The manometers are read 2 and 7 min. after tipping. The oxygen consumption in this 5 min. period is used in calculating enzyme activity. The amount of enzyme is adjusted so that the oxygen consumption is not higher than 8 μl./min. Under these conditions oxygen consumption is strictly proportional to the amount of enzyme used.

One methylene blue, or phenazine, unit is defined as the amount of enzyme capable of catalysing the consumption of 1 μl. of oxygen/min., when each dye respectively is used as hydrogen carrier.

With purified preparations (4·7 indophenol units/mg. of protein) the correspondence among the several defined units is: 1·0 indophenol unit ≡ 0·7 quinone unit ≡ 9·2 methylene blue units ≡ 28·5 phenazine units.

Purification procedure

Crude extract. The cells, harvested from the medium by centrifuging and washed with water, were frozen, ground with twice their weight of alumina-301 (Aluminium Co. of America) and the paste was extracted with a portion of 0·1 M-potassium phosphate buffer, pH 7·0, equal to three times the volume of the cells. The alumina and cell debris were then separated by centrifuging at 10 000 *g* for 15 min., and the supernatant was collected.

First ammonium sulphate fractionation. To 100 ml. of the crude cell extract obtained as described above, 17·6 g. of

solid $(NH_4)_2SO_4$ is added with stirring, and in small portions. Stirring is continued for 15 min. and the precipitated proteins are separated by centrifuging and discarded. A further 8·6 g. of $(NH_4)_2SO_4$ is added to the supernatant in the same manner. The precipitate formed is collected by centrifuging and dissolved in 0·1 M-sodium citrate buffer, pH 5·3, to give a solution containing about 25 indophenol units/ml.

Protamine treatment. To the solution obtained in the previous step is added an equal volume of 0·1 M-tris buffer, pH 8·8, and then 0·7 vol. of 0·5 % (w/v) protamine sulphate solution. After stirring for 10 min. the precipitate is separated by centrifuging and discarded. The supernatant is brought to pH 6·0 by the addition of 0·2 M-sodium acetate buffer, pH 4·0

Second ammonium sulphate fractionation. The solution obtained in the previous step is again fractionated with solid $(NH_4)_2SO_4$, the fraction separating between 25 and 30 % of saturation being collected by centrifuging and dissolved in 0·1 M-sodium citrate, pH 5·3, to give approx. 15 indophenol units/ml.

Isoelectric precipitation and ethanol fractionation. To the solution obtained in the previous step 0·2 vol. of twice-distilled water, and then 0·8 vol. of 0·2 M-sodium acetate buffer, pH 4·0, are added (final pH is now between 4·5 and 4·7). The inert material is separated by centrifuging, the solution is placed in the cold room at $-10°$ and its temperature is lowered until the first ice crystals begin to appear. Then 98 % (w/v) ethanol, cooled to $-20°$, is slowly added through a capillary pipette with stirring until a 10 % solution is obtained. At the end of the addition the temperature of the solution is about $-15°$ and the precipitate formed is separated by centrifuging at $-15°$; the precipitate is dissolved in 0·1 M-sodium citrate, pH 5·3, and dialysed against the same buffer (containing 0·01 M-EDTA) for 1 hr.

RESULTS

Purification and properties of the enzyme

Purification procedure. Table 1 shows the purification achieved by the procedure described above.

Absence of pyridine nucleotide moiety. The following evidence indicates that the lactic dehydrogenase from *P. pentosaceum* is independent of pyridine nucleotide coenzymes.

1. When purified preparations of specific activity 4·7 were dialysed against 0·025 M-sodium citrate, pH 5·3, for 7 hr. a 33 % decrease in activity occurred. The addition of DPN to the indophenol system in concentrations of up to 0·34 mM in the assay of these fractions did not restore the initial activity.

2. Relatively large amounts of lactic dehydrogenase (0·3 indophenol unit) failed to reduce either DPN or TPN in the presence of lactate. Similarly, DPNH was not oxidized by the addition of sodium pyruvate.

3. Malic dehydrogenase plus malate failed to bring about an increase in extinction at 340 mμ when added to either the purified *P. pentosaceum* lactic dehydrogenase preparations or to boiled juices (Kochsaft) obtained from these.

4. The dehydrogenase activity is not affected by 20 min. pre-incubation in 0·25 M-hydroxylamine or by the addition of this substance to the indophenol assay system in concentrations of 50 mM.

5. In the crude *P. pentosaceum* extracts DPNH disappeared rapidly, as measured by decrease in extinction at 340 mμ, and this decrease was identical both aerobically and anaerobically as well as in the presence or absence of pyruvate.

Specificity. Among several hydroxy acids tested, lactate was the only effective hydrogen donor observed: α-hydroxy*iso*butyric acid, β-hydroxybutyric acid and malic acid gave completely negative results. DPNH does not serve as hydrogen donor in the assay systems used.

A stereospecificity was observed and the relative velocities for the different lactates at a concentration of 1·67 mM in the assay system were as follows: racemic lactate, arbitrarily taken as 10; D-(−)-lactate, 9·3; L-(+)-lactate 3. The cause of the residual activity in the presence of L-(+)-lactate has not been determined.

Several dyes act as hydrogen acceptors, as indicated in the description of the assay methods. The relative efficiency of these dyes, throughout the course of purification, is nearly constant, as shown by the data of Table 2. Besides these, indigo tetrasulphonate (1·33 mM), toluidine blue (0·5 mg./ml.) and azure I (0·5 mg./ml.) were also effective, but the rates of consumption were 7, 8 and 17 % respectively of that obtained with methylene blue. Indigo disulphonate (1·33 mM), indigo trisulphonate (1·33 mM), Nile blue (0·5 mg./ml.) and cresyl violet (0·5 mg./ml.) were completely ineffective.

Table 1. *Purification of lactic dehydrogenase from* Propionibacterium pentosaceum

The activities were measured by the indophenol assay.

Fraction	Total indophenol units	Specific activity (units/mg. of protein)	Total protein (mg.)
Crude extract	1880	0·5	3980
First $(NH_4)_2SO_4$ fractionation	900	1·2	770
Protamine supernatant	642	1·0	650
Second $(NH_4)_2SO_4$ fractionation: 25–30 % saturation	216	2·8	78
Ethanol fractionation: 0–10 % ethanol	166	5·7	29

Table 2. *Comparison of activities observed with the various assay systems, shown with the several fractions obtained in the course of purification of* Propionibacterium pentosaceum *lactic dehydrogenase*

Specific activity is expressed in indophenol units/mg. of protein. The activities were assayed by the different methods as described in the text.

Fraction	(1) Specific activity	(2) Indophenol units/ml.	(3) Quinone units/ml.	(4) Phenazine units/ml.	(5) Methylene blue units/ml.	(3)/(2)	(4)/(2)	(5)/(2)
Crude extract	0·5	5·3	3·5	162	72	0·66	30·5	13·6
After first $(NH_4)_2SO_4$ fractionation	1·0	21·1	13·3	655	200	0·62	31·0	9·4
After protamine treatment and second $(NH_4)_2SO_4$ fractionation	2·6	12·7	9·0	380	116	0·70	30·0	9·2
After ethanol fractionation	4·1	12·3	8·7	374	112	0·70	30·4	9·1

Table 3. *Relation between the amount of dye reduced and pyruvate formed during lactate oxidation by* Propionibacterium pentosaceum *lactic dehydrogenase*

Variable amounts of 2:6-dichlorophenolindophenol (as indicated) were completely reduced by excess of lactate (20 μmoles) in the presence of 0·64 indophenol unit of lactic dehydrogenase (specific activity, 2·6), in a system containing 100 μmoles of potassium phosphate buffer, pH 7·3, in a total volume of 2 ml. The pyruvate in a portion of the medium was assayed directly and the results were corrected for blanks: (*a*) without dye; (*b*) without enzyme.

Expt. no.	Indophenol reduced (μmole)	Pyruvate formed (μmole)	Pyruvate / Dye
1	0·53	0·54	1·02
2	1·06	1·15	1·08
3	1·58	1·70	1·08

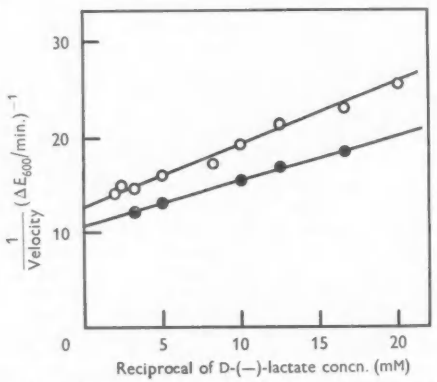

Fig. 1. Determination of the Michaelis constant of lactic dehydrogenase. The activity was measured by the indophenol assay with and without addition of ammonium sulphate, the concentration of D-(−)-lactate being varied as indicated. Enzyme of specific activity 4·7 was used. ○, Absence of ammonium salts; ●, presence of 10 mM-ammonium sulphate. pH 7·3 and temp. 26°.

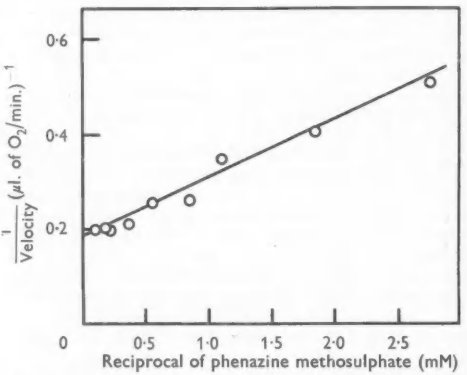

Fig. 2. Effect of varying phenazine methosulphate concentration on lactic dehydrogenase activity. Activity was measured by the phenazine methosulphate assay with solutions of varying concentrations of phenazine: 0·13 indophenol unit of purified enzyme of specific activity 4·7 was used per Warburg vessel. Temp. 30°.

Reaction products. The stoicheiometry of the reaction studied is the following:

$$\text{D-(}-\text{)-lactate} + \text{dye} \rightarrow \text{pyruvate} + \text{dye}\cdot H_2.$$

It was verified by paper chromatography (Cavallini, Frontali & Toschi, 1949) that pyruvic acid is the only keto acid formed under the assay conditions. The number of moles of pyruvate formed and the number of moles of reduced dye produced are nearly equal (Table 3).

Michaelis constants. The dependence of the rate of reduction of 2:6-dichlorophenolindophenol on the concentration of D-(−)-lactate is shown by the data presented in Fig. 1, from which it can be calculated that K_m for lactate in the absence of $(NH_4)_2SO_4$ is 51·6 μM at pH 7·3 and at 26°.

The affinity of the enzyme for the several hydrogen acceptors was also determined. In the manometric-assay conditions, the influence of phenazine

methosulphate on reaction rate is shown by the data of Fig. 2, and K_m for phenazine methosulphate is 640 μM. Under the same conditions, if methylene blue is used as acceptor, hardly any variation is observed in the velocity of lactate oxidation, when the dye concentration varies from 0·5 mM to 3·33 mM. Independence of reaction velocity on dye concentration was also observed with 2:6-dichlorophenolindophenol (14 μM–50 μM) and 1:2-naphthaquinone-4-sulphonate (0·5 mM–1·0 mM). Thus in the last three cases mentioned K_m is equal to or nearly equal to zero.

pH *dependence*. The effect of pH on reaction velocity was studied manometrically with methylene blue as the hydrogen acceptor. Fig. 3 shows that the optimum pH is 7·7.

Stability. The enzyme seemed to be fairly stable when kept at 0–4° in solutions of pH 5·3–5·8. The drop in activity was only 2% when solutions (5–10 indophenol units/ml.) in 0·1 M-sodium citrate, pH 5·3, containing 0·01 M-EDTA, were kept frozen for 1 month at −25°. Dilute enzyme (0·4 indophenol unit/ml.) in this same citrate buffer containing EDTA can be kept at 0° for 10 min. without any loss in activity. If, however, EDTA is not added an 8% drop in activity is observed.

The enzyme is thermolabile, activity being completely destroyed by boiling for 3 min., and only 15% of the activity remains after heating to 47° for 3 min., as measured by the indophenol assay.

Prosthetic group. The spectrum of the best preparation obtained is shown in Fig. 4. It shows general absorption in the visible range, and the shoulder at 420 mμ is due to contamination with cytochrome *b*, which is abundantly present in the crude preparations (Lara, 1959).

Many attempts to split the enzyme by the procedures of Horecker (1950) and of Theorell & Maelhy (1950) failed. The addition of 6% (w/v) perchloric acid to solutions of the enzyme in 20% (w/v) $(NH_4)_2SO_4$, to produce final concentrations of 6 mN-0·1 N, led to white precipitates which, when dissolved in 0·1 M-sodium citrate buffer, pH 5·3, showed from 0 to 60% of the original activity. Restoration of the activity was not obtained by addition of FMN, FAD or boiled juices obtained from purified enzyme.

Activators. Several ions activate the enzyme, as measured by the indophenol assay. The effect of the NH_4^+ ion is particularly striking (Table 4). This

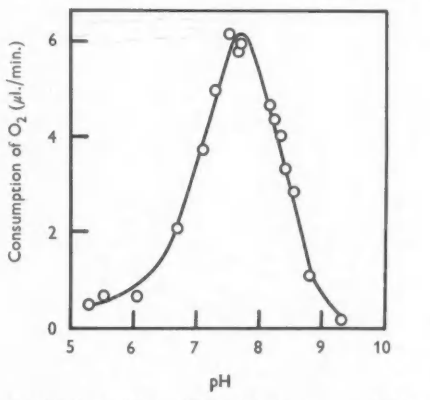

Fig. 3. Effect of pH on lactic dehydrogenase activity. Activities were measured by the methylene blue assay with 0·25 indophenol unit of purified enzyme of specific activity 4·1. Each Warburg vessel contained 100 μmoles of buffers of varying pH in place of the one regularly used (citrate, pH 5·3–6·0; phosphate, pH 6·7–7·7; tris-HCl, pH 7·7–8·5; carbonate, pH 8·85 and 8·3). Temp. 30°.

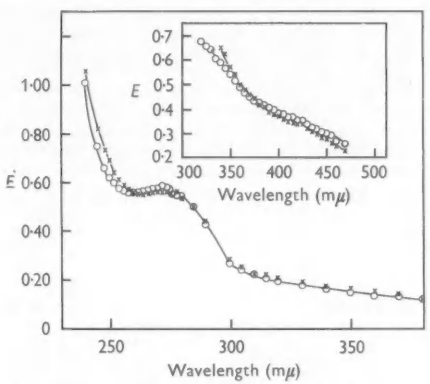

Fig. 4. Absorption spectra of purified lactic dehydrogenase. ○, Enzyme in equilibrium with air; ×, enzyme in the presence of sodium lactate. Main figure: solution of purified enzyme of specific activity 4·7, 1·6 indophenol units/ml.; insert: solution of purified enzyme of specific activity 5·5, concentration 16·4 indophenol units/ml.

Table 4. *Activation of* Propionibacterium pentosaceum *lactic dehydrogenase by ammonium salts*

The activities were measured by the indophenol assay, with enzyme of specific activity 3·1. The salts were included in the assay system in the concentration indicated. The activity in the absence of the salts was taken arbitrarily as 100.

Concen. (M)	Relative activity			
	$(NH_4)_2SO_4$	Na_2SO_4	NH_4Cl	NaCl
—	100	100	100	100
0·01	122	103	120	104
0·05	160	117	146	105
0·10	202	111	194	111
0·20	228	126	257	106
0·50	266	123	348	100

is not merely due to an increase in the ionic strength, since the activation caused by Na_2SO_4 or NaCl is either very small or non-existent as compared with the corresponding ammonium salts.

Table 5. *Activation of* Propionibacterium pentosaceum *lactic dehydrogenase by several substances*

The activities were measured as described in Table 4.

Activator	Concn. (mM)	Relative activity
None	—	100
Magnesium sulphate	5·0	126
Magnesium sulphate	10·0	129
Potassium cyanide	1·0	109
Potassium cyanide	3·3	125
Sodium fluoride	50·0	126
*iso*Nicotinic acid hydrazide	10·0	118
Potassium malonate	10·0	151

Table 6. *Action of some inhibitors on the dehydrogenation of lactate catalysed by purified preparations from* Propionibacterium pentosaceum

All experiments were conducted under the conditions described for the indophenol assay, the inhibitory substances being incorporated in the assay solution in the concentrations indicated. The observed result is therefore due to the action of the inhibitor during the 2 min. required to conduct the assay.

Inhibitors	Concn. (mM)	Inhibition (%)
Sodium pyruvate	0·3	21
	1·7	47
	8·3	74
Sodium oxalate	0·1	24
	0·2	43
	1·0	74
p-Chloromercuribenzoate	1·0	19
	2·0	64
	3·0	98
Monoiodoacetate	6·0	26
	8·2	44
Urethane	10·0	26
Amytal	10·0	44
Nembutal	10·0	9
	50·0	73
Quinacrine	0·1	30
	1·0	83
Chloroquine	1·0	20
Quinine	0·4	50
	1·0	72
Dicoumarol	0·02	16
	0·1	49
	1·0	96
Vitamin K_1	0·2	18
	5·0	59
Pentachlorophenol	0·1	19
	1·0	65
Thyroxine	0·2	19
Hydrazine	5·0	30
	20·0	64

The results shown in Fig. 1 indicate that the NH_4^+ ion alters the affinity of the enzyme for lactate.

Results obtained which indicate activation by other substances are presented in Table 5.

Metal components. The purified enzyme seems to be independent of metals for its action. Cyanide and fluoride activate (Table 5) whereas EDTA (0·1 M), 8-hydroxyquinoline (1 mM), sodium azide (1 mM) and 1:2-dihydroxybenzene-3:5-disulphonate (5 mM) do not decrease the activity when included in the indophenol assay system.

Action of inhibitors

Effect of pyruvate and oxalate. Table 6 shows the effect of pyruvate and oxalate on the lactic dehydrogenase activity. Kinetic studies of these inhibitions (Fig. 5) indicate that pyruvate inhibits non-competitively (Lineweaver & Burk, 1934), whereas oxalate acts uncompetitively (Burk, 1944).

Effect of sulphydryl reagents. Monoiodoacetate, *p*-chloromercuribenzoate and *o*-iodosobenzoate have strong inhibitory actions on the enzyme (Table 6). It was also found that the inhibition produced by both *p*-chloromercuribenzoate and *o*-iodosobenzoate is potentiated when they are preincubated with the enzyme in the presence of lactate (Table 7).

Heavy-metal ions (Hg^{2+}, Ag^+ and Cu^{2+} at 0·1 mM concentration) led to total inactivation of the enzyme.

Effect of narcotics. Urethane, Amytal and Nembutal are able to inhibit the dehydrogenase, as shown in Table 6.

Effect of flavin antagonists. Quinacrine (Atabrin), chloroquine and quinine have been described as

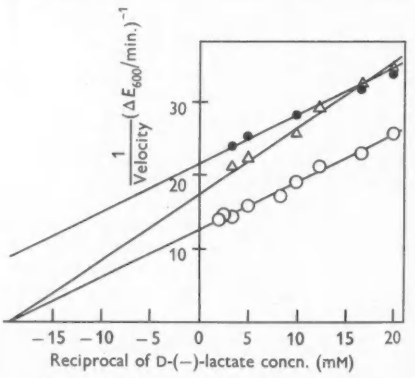

Fig. 5. Effect of sodium pyruvate and sodium oxalate on lactic dehydrogenase. Activity was measured at 26° by the indophenol assay with enzyme of specific activity 4·7 in the absence (○) and in the presence of 0·333 mM-sodium pyruvate (△) or 0·5 mM-sodium oxalate (●).

flavoprotein inhibitors (Haas, 1944; Wright & Sabine, 1944; Hellerman, Lindsay & Bovarnick, 1946). Data presented in Table 6 show that the preparations under study are sensitive to relatively low concentrations of these antimalarial drugs. These inhibitions could not be reversed by the addition of boiled juices prepared from the enzyme. Also FMN (0·1–1·0 μM) and FAD (0·1–1·0 μM) were ineffective. A reversion was obtained, however, on the addition of perchloric acid extracts, which were prepared as described in the Materials and Methods section. The results obtained are shown in Table 8. If the extracts were irradiated for 30 min. with ultraviolet light, their capacity for reversing the inhibition was completely lost (Table 8).

The inhibition by quinine is uncompetitive (Burk, 1944) (Fig. 6).

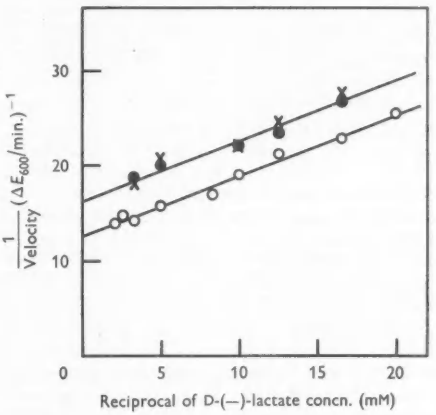

Fig. 6. Effect of dicoumarol and quinine on lactic dehydrogenase. Activity was measured at 26° by indophenol assay with enzyme of specific activity 4·7 in the absence (○) and presence of 40 μM dicoumarol (●) or 100 μM-quinine (×).

Table 7. *Inhibition of lactic dehydrogenase by p-chloromercuribenzoate and o-iodosobenzoate, in the presence and absence of lactate*

To 0·6 unit of lactic dehydrogenase (specific activity 3·5) in 0·1 ml. were added 0·8 ml. of 0·5% bovine plasma-albumin solution, the inhibitors and lactate to obtain the concentrations indicated. The final volume was made to 1 ml. in all cases by addition of water. Incubation time: 5 min.; temperature: 0°. The activity remaining at the end of incubation was determined by the indophenol assay.

Inhibitor	Concn. (mM)	Inhibition (%) With 50 mM-lactate	Inhibition (%) Without lactate
p-Chloromercuribenzoate	0·2	33	16
o-Iodosobenzoate	1·0	30	5
	2·0	36	2
	5·0	54	36

Table 8. *Inhibition of lactic dehydrogenase by quinacrine and quinine, and reversal by perchloric acid extracts*

The activity determinations were made by the indophenol assay, the inhibitors and perchloric acid extract (see Materials and Methods section) being added to the assay reagents before enzyme addition.

Inhibitor	Concn. (mM)	Perchloric acid extract units	Inhibition (%)
Quinacrine	0·1	—	38
	0·1	0·3	23
	0·1	0·6	13
	0·1	0·3*	38
Quinine	0·2	—	36
	0·2	1·3	12
	0·4	—	41
	0·4	1·3	16

* This extract was irradiated with ultraviolet light for 30 min. (Mineralight SL-2537, Ultraviolet Products Inc., South Pasadena, Calif., U.S.A.).

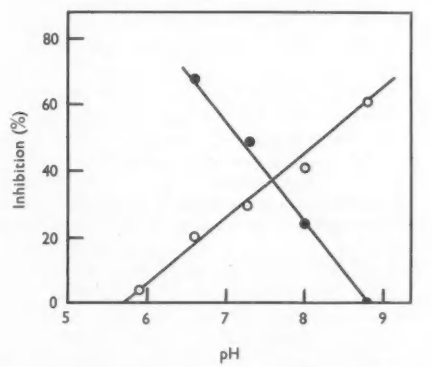

Fig. 7. Effect of pH on the inhibition of lactic dehydrogenase by quinacrine and dicoumarol. All experiments were made with enzyme of specific activity 4·7. The data for pH 7·3 were obtained by the regular indophenol assay. For other pH values, phosphate, pH 5·9 and 6·6, and tris buffer, pH 8·0 and 8·8, were substituted for the phosphate solution employed normally in the assay, the buffer concentration being always the same. For either inhibitor the concentration was 100 μM and percentage inhibition at a given pH was expressed by taking arbitrarily the activity in the absence of inhibitor as 100. The actual extinction changes per minute in the absence of inhibitor were: pH 5·9, 0·031; pH 6·6, 0·050; pH 7·3, 0·052; pH 8·0, 0·044; pH 8·8, 0·026. ○, Quinacrine; ●, dicoumarol.

Effect of dicoumarol and vitamin K_1. The sensitivity of the dehydrogenase to dicoumarol and vitamin K_1 is shown in Table 6. A kinetic study of the dicoumarol inhibition was carried out (Fig. 6) and the results obtained indicate that dicoumarol acts uncompetitively (Burk, 1944).

Menadione in concentrations up to 0·5 mM did not show any effect on enzyme activity, either in the presence or absence of dicoumarol. In contrast, vitamin K_1 and dicoumarol seem to interact in some manner, for it was observed that the combined inhibition produced by vitamin K_1 (0·44 mM) and dicoumarol (0·1 mM) was only 41 %, whereas either of these substances alone would inhibit 31 % and 48 % respectively.

Effect of other substances. Data in Table 6 show that the preparations are inhibited by pentachlorophenol, thyroxine and hydrazine. Several other substances were also found to be without effect on the enzyme activity; among these are 2:6-dichlorophenolindophenol (when the concentration in the assay system was not greater than 0·1 mM), 2:4-dinitrophenol, adenine, FAD, FMN, ADP, ATP, hydroxylamine and the chelating agents mentioned under metal components above.

Effect of pH *on quinacrine and dicoumarol inhibitions.* Fig. 7 shows the pH dependency of the inhibition produced by quinacrine and dicoumarol.

DISCUSSION

Although much effort was spent in searching for purification methods, the best method obtained resulted in only a 15-fold enrichment in activity. The relation of the activities observed with the various electron acceptors is nearly constant throughout the purification. This might indicate that only one enzyme is necessary for the reduction of all dyes. It might also be possible that something like the 'electron-transferring flavoprotein' of Crane & Beinert (1956) is operative in *P. pentosaceum*. This hypothesis is attractive because it could explain the difficulty experienced in purifying the enzyme. No evidence for this has been obtained, however.

The enzyme is very likely independent of freely dissociable coenzymes. The evidence presented by Barker & Lipmann (1944), based on thermodynamic considerations, had already led to this conclusion, which is supported by the results presented in this paper. The enzyme is specific for D-(−)-lactate and in this respect resembles that found in *Escherichia coli* (Szulmajster, Grunberg-Manago & Delavier-Klutchko, 1953) and differs from that found in muscle (Kubowitz & Ott, 1943) and yeast (Boeri, Cutolo, Luzzati & Tosi, 1955).

The highest reaction velocity is observed with phenazine methosulphate as the acceptor, and the following relationship was found relative to 1:2-naphthaquinone sulphonate (1·0): phenazine methosulphate, 3·6; 2:6-dichlorophenolindophenol, 1·4; methylene blue, 1·2.

The activating action of NH_4^+ ion seems to alter the affinity of the enzyme towards lactate. Similar phenomena have been described in the literature for other enzymes (Mahler, Raw, Molinari & Amaral, 1958; Huennekens, Basford & Gabrio, 1955; Carpenter, 1951). The effect observed with the other activators might be similar to that of ethylenediaminetetra-acetic acid, i.e. they might act by withdrawing heavy metals which are deleterious to the enzyme.

All attempts to obtain evidence for the existence of flavins as prosthetic groups in this enzyme failed. The presence of flavins is by no means excluded, and the absence of a typical flavin spectrum might easily be due to interference by the presence of the haemoprotein, as shown with other enzymes (Horecker, 1950; Mahler, Sarkar, Vernon & Alberty, 1952).

The data presented indicate that the lactate dehydrogenation catalysed by the preparations studied depends on SH groups. The fact that the enzyme incubated with lactate is more sensitive to *p*-chloromercuribenzoate than when incubated with pyruvate might mean that more free SH groups are produced in the first case, and suggests that the SH groups might be involved in electron transport in this case.

The reversal of the inhibition effected by quinine and quinacrine by addition of perchloric acid extracts obtained from the purified enzyme indicates the necessity of a cofactor. The nature of the substance involved is unknown, but its character is unlikely to be inorganic since ultraviolet irradiation and boiling seem to destroy it. These results are also important because they exclude the possibility that the inhibition observed might be due to a chelating effect of either quinine or quinacrine. The possibility that the extracts do contain a flavin of constitution different from FAD or FMN is by no means eliminated.

Few soluble enzymes are known to be inhibited by dicoumarol (Wosilait & Nason, 1954; Martius, 1955; Martius & Marki, 1957; Mahler *et al.* 1958) and the preparations here studied constitute an addition to this group. Through the work performed by Martius & Nitz-Litzow (1953, 1954*a*, *b*) and by Wosilait & Nason (1954) it became evident that dicoumarol acts by virtue of its quinone structure. These observations, and the ones reported in this paper in connexion with the interaction between dicoumarol and vitamin K_1, make it probable that the enzymes of *P. pentosaceum* catalysing the reduction of 2:6-dichlorophenolindophenol by lactate may require a cofactor of a quinone nature.

The results obtained in the study of the pH effect on dicoumarol and quinacrine inhibition permit the formulation of a hypothesis about the binding of these substances on the enzyme surface. Thus according to the theory of Dixon (1953), and since dicoumarol is an acid, the negative slope observed in curve 1 of Fig. 7 might be interpreted as indicating a union of dicoumarol with some positive group in the enzyme. Similarly, quinacrine might be bound to a negative group.

The preparations studied resemble other known lactic dehydrogenases (Quastel & Wooldridge 1928; Etling, 1953) in being inhibited by oxalate and by narcotics; hydrazine inhibits, but *iso*nicotinic acid hydrazide and hydroxylamine are without action.

SUMMARY

1. Preparations capable of oxidizing lactate to pyruvate in the presence of suitable hydrogen acceptors were obtained from *Propionibacterium pentosaceum*, by grinding frozen cells with alumina, followed by extraction with dilute phosphate buffer.

2. Several assay methods are described and a purification procedure is presented whereby a 15-fold concentration of the activity is obtained.

3. The requirements for half-saturation of the enzyme were found to be: D-(−)-lactate (51·6 μM), phenazine methosulphate (0·64 mM). The enzyme had infinite affinity for methylene blue, 2:6-dichlorophenolindophenol and 1:2-naphthaquinone-4-sulphonate.

4. The optimum pH for lactate oxidation was found to be 7·7 at 30°.

5. The preparations are activated by NH_4^+, CN^-, F^-, Mg^{2+} ions and malonate and are thermolabile.

6. The preparations are inhibited by pyruvate, oxalate, thiol reagents, narcotics, quinacrine, chloroquine, quinine, dicoumarol, vitamin K_1, pentachlorophenol, thyroxine and hydrazine.

7. The inhibition of quinacrine is reversed by perchloric acid extracts obtained from the purified enzyme, but not by boiled juices prepared from it or by flavinadenine dinucleotide or flavin mononucleotide.

8. The effect of pH on the inhibition by quinacrine and dicoumarol was studied.

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REFERENCES

Barker, H. A. & Lipmann, F. (1944). *Arch. Biochem.* **4**, 361.
Basford, R. E. & Huennekens, F. M. (1955). *J. Amer. chem. Soc.* **77**, 3873.
Boeri, E., Cutolo, E., Luzzati, M. & Tosi, L. (1955). *Arch. Biochem. Biophys.* **56**, 487.
Burk, D. (1944). Quoted by Ebersole, R. E., Guttentag, C. & Wilson, P. W. (1944). *Arch. Biochem.* **3**, 399.
Carpenter, F. H. (1951). *Acta chem. scand.* **5**, 406.
Cavallini, D., Frontali, N. & Toschi, G. (1949). *Nature, Lond.*, **163**, 568.
Crane, F. L. & Beinert, H. (1956). *J. biol. Chem.* **218**, 717.
Dixon, M. (1953). *Biochem. J.* **55**, 161.
Etling, N. (1953). *Bull. Soc. Chim. biol., Paris*, **35**, 1129.
Friedemann, T. E. & Haugen, G. E. (1943). *J. biol. Chem.* **147**, 415.
Gornall, A. G., Bardawill, C. J. & David, M. M. (1949). *J. biol. Chem.* **177**, 751.
Haas, E. (1944). *J. biol. Chem.* **155**, 321.
Hellerman, L., Lindsay, A. & Bovarnick, M. (1946). *J. biol. Chem.* **163**, 553.
Horecker, B. L. (1950). *J. biol. Chem.* **183**, 593.
Huennekens, F. M., Basford, R. E. & Gabrio, B. W. (1955). *J. biol. Chem.* **213**, 951.
Kubowitz, F. & Ott, P. (1943). *Biochem. Z.* **314**, 94.
Lara, F. J. S. (1954). *Ciênc. e Cult.* **6**, 171.
Lara, F. J. S. (1959). *Biochim. biophys. Acta*, **33**, 565.
Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
McIlwain, H. (1948). *J. gen. Microbiol.* **2**, 288.
Mahler, H. R., Raw, I., Molinari, R. & Amaral, D. F. (1958). *J. biol. Chem.* **233**, 230.
Mahler, H. R., Sarkar, N. K., Vernon, L. P. & Alberty, R. A. (1952). *J. biol. Chem.* **199**, 585.
Martius, C. (1955). *Angew. Chem.* **67**, 161.
Martius, C. & Marki, F. (1957). *Biochem. Z.* **329**, 450.
Martius, C. & Nitz-Litzow, D. (1953). *Biochim. biophys. Acta*, **12**, 134.
Martius, C. & Nitz-Litzow, D. (1954*a*). *Biochim. biophys. Acta*, **13**, 152.
Martius, C. & Nitz-Litzow, D. (1954*b*). *Biochim. biophys. Acta*, **13**, 289.
Quastel, J. H. & Wooldridge, W. R. (1928). *Biochem. J.* **22**, 689.
Singer, T. P. & Kearney, E. B. (1957). In *Methods of Biochemical Analysis*, vol. 4, p. 307. Ed. by Glick, D. New York: Interscience Publishers Inc.
Szulmajster, J., Grunberg-Manago, M. & Delavier-Klutchko, C. (1953). *Bull. Soc. Chim. biol., Paris*, **35**, 1381.
Theorell, H. & Maelhy, A. C. (1950). *Acta chem. scand.* **4**, 422.
Wosilait, W. D. & Nason, A. (1954). *J. biol. Chem.* **208**, 785.
Wright, C. I. & Sabine, J. C. (1944). *J. biol. Chem.* **155**, 315.

Biochem. J. (1960) 75, 66

The Mechanism of the Reversible Aerobic Inhibition of Glycolysis in a Pea-Seed Extract

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Previous studies established that a partially purified extract from pea seeds contained a complete Embden–Meyerhof–Parnas glycolytic system (Turner, 1954, 1957; Hatch & Turner, 1958). Glycolytic activity was inhibited by aerobic conditions (Turner & Mapson, 1958; Hatch & Turner, 1959) and some properties of this effect were similar to those of the Pasteur effect of intact tissues. Further investigations (Hatch & Turner, 1959) showed that the aerobic inhibition of glycolysis in the pea-seed extracts was due to the reversible inactivation of glyceraldehyde 3-phosphate dehydrogenase. Balazs & Richter (1958*a*, *b*) found that a similar mechanism was partly responsible for the aerobic inhibition of glycolysis by a brain-mitochondrial preparation.

The present investigation is concerned with the mechanism of the reversible aerobic inhibition of glyceraldehyde 3-phosphate dehydrogenase and the oxidation of protein sulphydryl groups and reduction of protein disulphide groups in the pea-seed extracts.

EXPERIMENTAL

Preparation of the pea-seed extract

The partially purified pea-seed extract was prepared as described by Turner (1957), except that extracts used for glycolytic studies were dialysed against 25 mM-phosphate buffer (KH_2PO_4–Na_2HPO_4) at pH 6·5. The extract contained approx. 45 mg. of protein/ml. Extracts used for the study of protein disulphide (S–S)-group reduction or the activation of glyceraldehyde 3-phosphate dehydrogenase were incubated in air for 2 hr. at 30° after dialysis. This treatment decreased the protein sulphydryl (SH)-group content from approx. 25 μg.equiv. of SH/g. of protein to approx. 15 μg.equiv. of SH/g. of protein.

Materials

Fructose, glucose 6-phosphate (G 6-P), adenosine triphosphate (ATP), diphosphopyridine nucleotide (DPN), reduced diphosphopyridine nucleotide (DPNH), triphosphopyridine nucleotide (TPN), reduced glutathione (GSH), cysteine hydrochloride and *p*-chloromercuribenzoate were commercial products as described previously (Hatch & Turner, 1958, 1959). Oxidized glutathione (GSSG) was obtained from C. F. Boehringer und Söhne, Mannheim, Germany, and cystine and fructose 1:6-diphosphate (FDP) were from L. Light and Co. Ltd.

Glucose 1-phosphate (G 1-P) was prepared by an unpublished method of Professor C. S. Hanes and Dr R. Hill and pea-seed glyceraldehyde 3-phosphate dehydrogenase was prepared according to Hageman & Arnon (1955). Solutions of G 1-P, G 6-P and FDP were standardized as described previously (Hatch & Turner, 1958).

Analytical methods

Glyceraldehyde 3-phosphate dehydrogenase activity. Glyceraldehyde 3-phosphate dehydrogenase activity in the pea-seed extract was determined as described previously (Hatch & Turner, 1959). The activity of the pea-seed glyceraldehyde 3-phosphate dehydrogenase preparation was determined by the method of Warburg & Christian (1939).

Cystine reductase and oxidized glutathione reductase activity. Cystine reductase and GSSG reductase were assayed as described by Romano & Nickerson (1954) and Racker (1955) respectively.

Pyridine nucleotide transhydrogenase activity. Two spectrophotometric methods were used to determine whether pyridine nucleotide transhydrogenase was present in the pea-seed extract. The extract contained a DPN-specific alcohol dehydrogenase and a TPN-specific GSSG reductase. TPN reduction was measured in a reaction mixture containing 0·3 ml. of the pea-seed extract (containing alcohol dehydrogenase); Na_2HPO_4–KH_2PO_4 buffer (pH 6·8), 50 μmoles; ethanol, 0·1 ml.; DPN, 0·05 μmole; TPN, 0·6 μmole; total vol., 2·8 ml. DPNH oxidation was measured in a reaction mixture containing 0·3 ml. of the pea-seed extract (containing GSSG reductase); Na_2HPO_4–KH_2PO_4 buffer (pH 7·0), 50 μmoles; GSSG, 12 μmoles; TPN, 0·05 μmole; DPNH, 0·65 μmole; total vol., 2·8 ml. Both assays were carried out at 26°. No evidence was obtained for pyridine nucleotide transhydrogenase activity.

Assay of SH *groups.* The method used to estimate protein SH groups was described previously (Hatch & Turner, 1958).

The SH groups of glutathione were determined by amperometric titration with $AgNO_3$ (Benesch & Benesch, 1945). Cysteine SH groups were determined by the method of MacDonnell, Silva & Feeney (1951).

Determination of protein. The protein content of the pea-seed extract and the pea-seed glyceraldehyde 3-phosphate dehydrogenase preparation was estimated by the spectrophotometric method of Warburg & Christian (1941).

Enzyme studies

Glycolytic activity was determined as described previously (Hatch & Turner, 1958). Other reaction mixtures were incubated at 30° in Thunberg tubes and anaerobic

conditions were established by alternately evacuating and flushing with O_2-free N_2. Spectrophotometric studies were conducted at 26°.

RESULTS

Relationship of protein SH*-group concentration and glycolytic activity*

It was shown previously that glycolytic activity in the pea-seed extract was related to the extent of oxidation of the constituent protein SH groups (Hatch & Turner, 1959). When the reaction mixtures were incubated in air the concentration of protein SH groups either increased or decreased (compared to the initial level of 25 μg. equiv. of SH/g. of protein) depending on the amount of DPN added. When the aerobic glycolytic activity was plotted against protein SH-group content a linear relationship was obtained (Fig. 1). These experiments indicated that mechanisms for the oxidation of protein SH groups and the reduction of protein S–S groups were operating in the enzyme digests.

Oxidation of protein SH *groups and the inhibition of glyceraldehyde* 3*-phosphate dehydrogenase*

When the pea-seed extract was incubated in air there was a decrease in the protein SH-group content (Table 1). The addition of ethylenediaminetetra-acetic acid (EDTA) (final concn. 0·15 mM) inhibited this aerobic oxidation by 80%. However, concentrations of DPN which prevented the aerobic inhibition of glycolysis in the pea-seed extracts (Hatch & Turner, 1959) had little influence on the decrease in protein SH groups. Glyceraldehyde 3-phosphate dehydrogenase activity in the pea-seed extract was affected in a similar manner probably through the oxidation of essential SH groups on the enzyme molecule (Table 2). EDTA (0·3 mM) reduced the rate of aerobic inactivation of glyceraldehyde 3-phosphate dehydrogenase activity but DPN (0·5 mM) had no effect.

Mandels (1956) reported the direct enzymic oxidation by molecular O_2 of the SH groups of cysteine and GSH by an extract from fungi. In the present investigation it was found that the addition of the pea-seed extract to solutions of cysteine or GSH in

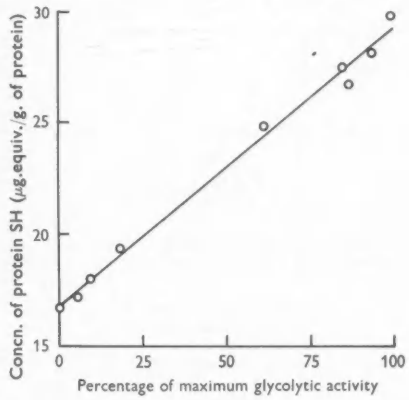

Fig. 1. Relationship of the protein SH-group concentration to aerobic glycolytic activity. Reaction mixtures contained: pea-seed extract, 1·3 ml.; Na_2HPO_4–KH_2PO_4 buffer (pH 6·5), 25 μmoles; G 1-P, 22 μmoles; fructose, 66 μmoles; $MgCl_2$, 18 μmoles; ATP, 0·23 μmole; total vol., 2·3 ml. DPN concentration was varied from 0 to 0·31 mM to give different steady rate glycolytic activities. Gas phase, air.

Table 1. *Effect of ethylenediaminetetra-acetic acid and diphosphopyridine nucleotide on the aerobic oxidation of protein* SH *groups in the pea-seed extract*

Reaction mixtures contained: Expt. 1: pea-seed extract, 1·5 ml.; Na_2HPO_4–KH_2PO_4 buffer (pH 7·0), 37 μmoles; $MgCl_2$, 18 μmoles; total vol., 2·3 ml. Expt. 2: pea-seed extract, 3·5 ml.; Na_2HPO_4–KH_2PO_4 buffer (pH 7·0), 87 μmoles; $MgCl_2$, 36 μmoles; total vol., 7·2 ml. EDTA (0·15 mM) and DPN (0·4 mM) were added as shown. Gas phase, air.

Expt. no.	Additions	Time (hr.)	Concn. of protein SH groups (μg.equiv./g. of protein)
1	—	0	23·6
	—	1	16·3
	EDTA	1	22·1
2	—	0	26·0
	—	2	16·9
	DPN	2	18·5

Table 2. *Effect of ethylenediaminetetra-acetic acid and diphosphopyridine nucleotide on the aerobic inhibition of glyceraldehyde* 3*-phosphate dehydrogenase in the pea-seed extract*

Reaction mixture contained: pea-seed extract, 2·5 ml.; Na_2HPO_4–KH_2PO_4 buffer (pH 7·0), 92 μmoles; total vol., 6·1 ml. EDTA (0·3 mM) and DPN (0·5 mM) were added as indicated. Gas phase, air.

Additions	Time (min.)	Glyceraldehyde 3-phosphate dehydrogenase activity (ΔE/min.)
None	0	0·142
EDTA	0	0·170*
None	70	0·043
EDTA	70	0·114
DPN	70	0·046

* EDTA caused some activation of glyceraldehyde 3-phosphate dehydrogenase.

phosphate buffer brought about no increase in the rate of oxidation of the SH groups of these compounds.

Reduction of protein S–S *groups*

The observation that protein S–S groups were reduced during glycolysis suggested that DPNH (generated during glycolysis) may have been responsible for the reduction. When substrate levels of DPNH were added the protein SH-group concentration in the pea-seed extract increased from 14·0 μg. equiv. to 27·2 μg.equiv./g. of protein in 2 hr. (Table 3). Protein S–S groups were also reduced when DPNH was produced in the reaction mixtures. DPNH was generated by glyceraldehyde 3-phosphate dehydrogenase by adding either fructose plus G 1-P or FDP together with a catalytic amount of DPN; these treatments increased the protein SH-group concentration from 15·5 μg.-equiv. to 30·3 (in 4 hr.) and 28·0 (in 2 hr.) μg.-equiv./g. of protein respectively. Fluoride was added to inhibit glycolysis at the 3-phosphoglyceric acid stage. The alcohol dehydrogenase present in the pea-seed extract was also used to generate DPNH and, on the addition of ethanol, there was an increase in the protein SH-group content. TPNH, produced by the G 6-P dehydrogenase present in the pea-seed extract, was also effective in reducing the protein S–S groups. As pyridine nucleotide transhydrogenase activity could not be detected in the pea-seed extract it is probable that the protein S–S reducing system utilized DPNH or TPNH directly.

There was no reduction of protein S–S groups when a pea-seed glyceraldehyde 3-phosphate dehydrogenase preparation was incubated with DPNH (Table 4). When a small quantity (equivalent to 8 mg. of protein) of the partially purified

Table 3. *Increase of protein* SH *groups in pea-seed extract by reduced diphosphopyridine nucleotide or by systems producing reduced di- or tri-phosphopyridine nucleotide*

Reaction mixtures contained: Expt. 1: pea-seed extract, 1·5 ml.; Na_2HPO_4–KH_2PO_4 buffer (pH 7·0), 37 μmoles; $MgCl_2$, 18 μmoles; ATP, 0·75 μmole; DPNH as indicated; total vol., 2·5 ml. Expt. 2: pea-seed extract, 1 ml.; Na_2HPO_4–KH_2PO_4 buffer (pH 7·0), 25 μmoles; $MgCl_2$, 18 μmoles; ATP, 0·75 μmole; total vol., 2·2 ml. Additions to produce DPNH or TPNH are shown in the table. Gas phase, N_2.

Expt. no.	Additions	Time (hr.)	Concn. of protein SH groups* (μg.equiv./g. of protein)
1	DPNH (5 mM)	0	14·0
	DPNH (5 mM)	2	27·2
2	None	0	15·5
	None	2	15·5
	Fructose (22 mM); G 1-P (13 mM); DPN (mM); fluoride (4·5 mM)	4	30·3
	FDP (25 mM); fluoride (4·5 mM); DPN (mM)	2	28·0
	Ethanol (0·24 M); DPN (mM)	2	22·3
	G 6-P (22 mM); TPN (mM)	2	26·4

* There was no significant increase in concentration of SH groups when either substrate or pyridine nucleotide was omitted.

Table 4. *Enzymic reduction of protein* S–S *groups in a pea-seed glyceraldehyde 3-phosphate dehydrogenase preparation*

Reaction mixtures contained: pea-seed glyceraldehyde 3-phosphate dehydrogenase preparation (containing 112 mg. of protein); Na_2HPO_4–KH_2PO_4 buffer (pH 7·0), 26 μmoles; $MgCl_2$, 18 μmoles; ATP, 1·5 μmoles; total vol., 3·0 ml. Pea-seed extract (0·25 ml., containing 8 mg. of protein) and DPNH (12 μmoles) were added as indicated. Gas phase, N_2.

Additions	Time (hr.)	Concn. of SH groups in glyceraldehyde 3-phosphate dehydrogenase preparation* (μg.equiv./g. of protein)
DPNH	0	0·45
DPNH	1·5	0·45
Pea-seed extract + DPNH	0	0·67
Pea-seed extract + DPNH	1·5	5·02
Boiled pea-seed extract + DPNH	0	0·48
Boiled pea-seed extract + DPNH	1·5	0·72
Pea-seed extract	0	0·65
Pea-seed extract	1·5	0·83

* Corrected for the protein SH of the pea-seed extract.

pea-seed extract was added to the system there was a marked increase in the protein SH-group content of the glyceraldehyde 3-phosphate dehydrogenase preparation. There was very little increase when DPNH was omitted or when the partially purified pea-seed extract was boiled. It was concluded that a heat-labile component, presumably an enzyme which catalysed the reduction of protein S–S groups, was present in the pea-seed extract.

The rate of reduction by DPNH of protein S–S groups in the pea-seed extract was only slightly decreased when the extract was dialysed for 20 hr. instead of 2·5 hr. (Fig. 2). A DPN-specific cystine reductase (Romano & Nickerson, 1954) and a TPN-specific GSSG reductase (Mapson & Goddard, 1951; Conn & Vennesland, 1951) have been identified in pea-seed extracts. The preparation used in the present studies contained both enzymes and their possible role in the reduction of protein S–S groups is discussed subsequently.

Activation of glyceraldehyde 3-phosphate dehydrogenase by reduced diphosphopyridine nucleotide

When the pea-seed extract was incubated in air there was a decrease in glyceraldehyde 3-phosphate dehydrogenase activity (Fig. 3). This decrease was largely prevented if the extract was incubated under anaerobic conditions (Hatch & Turner, 1959). In the presence of a system which generated DPNH, glyceraldehyde 3-phosphate dehydro-

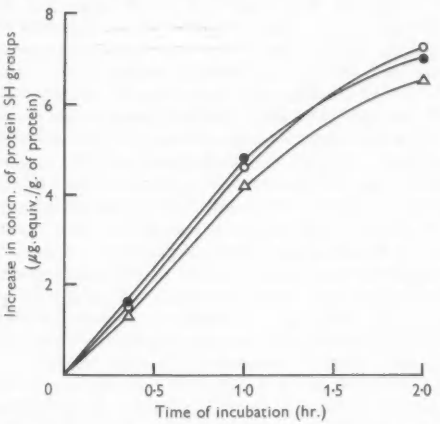

Fig. 2. Effect of dialysis on the protein S–S-group reducing activity of the pea-seed extract. Reaction mixtures contained: pea-seed extract, 1·5 ml. (○, dialysed for 2·5 hr.; ●, dialysed for 10 hr.; △, dialysed for 20 hr.); Na_2HPO_4–KH_2PO_4 buffer (pH 7·0), 36 μmoles; $MgCl_2$, 18 μmoles; ATP, 1·2 μmoles; DPNH, 10 μ-moles; total vol., 2·5 ml. Experiments were conducted under anaerobic conditions.

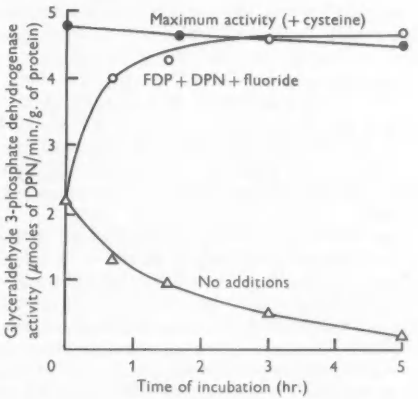

Fig. 3. Reversal of the aerobic inhibition of glyceraldehyde 3-phosphate dehydrogenase in the pea-seed extract by a DPNH-producing system and cysteine. Reaction mixtures of the following composition were incubated in air: pea-seed extract, 1·5 ml.; Na_2HPO_4–KH_2PO_4 buffer (pH 6·5), 38 μmoles; $MgCl_2$, 36 μmoles; ATP, 1·5 μmoles; total vol., 6 ml. Additions (as shown) were: FDP, 10 μmoles; DPN, 1·5 μmoles; fluoride, 36 μmoles. Samples of the reaction mixture to which no additions had been made were used to determine glyceraldehyde 3-phosphate dehydrogenase activity in the presence of cysteine (final concn. 5 mM).

Table 5. *Enzyme-mediated activation of pea-seed glyceraldehyde 3-phosphate dehydrogenase by reduced diphosphopyridine nucleotide*

Reaction mixtures contained: Na_2HPO_4–KH_2PO_4 buffer (pH 7·0), 35 μmoles; $MgCl_2$, 18 μmoles; ATP, 1·1 μmoles; DPNH, 11 μmoles; total vol., 2·2 ml. Pea-seed extract (0·15 ml. containing 5·5 mg. of protein) and the pea-seed glyceraldehyde 3-phosphate dehydrogenase preparation (containing 5·1 mg. of protein) were added as indicated. Gas phase, N_2.

Additions	Glyceraldehyde 3-phosphate dehydrogenase activity* (10^3 ΔE/min.) 0 hr.	2 hr.
Glyceraldehyde 3-phosphate dehydrogenase prepn.	2 (110)	0 (116)
Pea-seed extract	3 (24)	16 (27)
Glyceraldehyde 3-phosphate dehydrogenase prepn. + pea-seed extract	0 (136)	66 (146)
Glyceraldehyde 3-phosphate dehydrogenase prepn. + boiled pea-seed extract	4 (120)	3 (124)

* Maximum glyceraldehyde 3-phosphate dehydrogenase activity (assayed with cysteine, final concn. 5 mM) is quoted in parentheses.

genase activity in the pea-seed extract increased rather than decreased when the enzyme digests were incubated in air. When compared with control assays conducted in the presence of cysteine, glyceraldehyde 3-phosphate dehydrogenase was completely activated after approx. 2 hr. DPNH was generated by the addition of FDP, DPN and fluoride. Glyceraldehyde 3-phosphate dehydrogenase was not activated when either FDP or DPN was omitted.

The pea-seed glyceraldehyde 3-phosphate dehydrogenase preparation was substantially inactive unless cysteine was added. When DPNH alone was added to the glyceraldehyde 3-phosphate dehydrogenase preparation there was no increase in activity (Table 5). The figures for the maximum glyceraldehyde 3-phosphate dehydrogenase activity obtained when the assay was conducted in the presence of cysteine are also shown in Table 5. On the addition of a small quantity of the pea-seed extract (equivalent to 5·5 mg. of protein) there was a substantial increase in glyceraldehyde 3-phosphate dehydrogenase activity above the increase due to activation of the enzyme present in the pea-seed extract. The boiled pea extract was ineffective. No activation of the enzyme was observed when DPNH was omitted. It appeared that the reactivation of glyceraldehyde 3-phosphate dehydrogenase was catalysed by an enzyme contained in the pea-seed extract with DPNH functioning as hydrogen donor.

DISCUSSION

The present investigation has shown that mechanisms exist in the pea-seed extract for the oxidation of protein SH groups and for the enzymic reduction of protein S–S groups. Similarly glyceraldehyde 3-phosphate dehydrogenase was oxidatively inactivated and the oxidized form of the enzyme was also reactivated, presumably through enzymic reduction of S–S groups. On the basis of the observed activity of glyceraldehyde 3-phosphate dehydrogenase in the pea-seed extract, it was calculated that the essential SH groups of the enzyme probably amounted to less than 2% of the protein SH-group fraction which was directly related to the rate of glycolysis. This figure was derived from data of the activity of pea-seed glyceraldehyde phosphate dehydrogenase (Hageman & Arnon, 1955), assuming that the number of essential SH groups per molecule of the pea-seed enzyme was similar to that of the mammalian enzyme (Velick, 1953). The relationship obtained between glycolytic activity and the fraction of protein SH groups oxidized and reduced under aerobic conditions suggested that both the glyceraldehyde 3-phosphate dehydrogenase and non-glyceraldehyde 3-phosphate dehydrogenase SH groups contained in this fraction had similar oxidation–reduction properties.

There was no evidence in the present investigation that the oxidation of protein SH groups was enzymic. The inhibition by EDTA of the oxidation of both protein SH groups and glyceraldehyde 3-phosphate dehydrogenase indicated that the oxidation may have been a non-enzymic, metal-catalysed process. Fromageot (1951) showed that the non-enzymic oxidation of SH groups by oxygen was accelerated by traces of heavy-metal ions. Haugaard, Hess & Itskovitz (1957) found that this oxidation was inhibited by EDTA. The direct enzymic oxidation of non-protein SH groups by molecular oxygen has been reported by Mandels (1956) with extracts of fungal spores and by Neufeld, Green, Latterell & Weintraub (1958) with culture filtrates of fungi. Similar enzymes could mediate the oxidation of protein SH groups either directly or indirectly in the presence of catalytic amounts of cysteine, GSH or other SH-group compounds, but activity of this type could not be detected in the pea-seed extract.

Previous experiments (Hatch & Turner, 1959) and those reported in the present investigation showed that protein S–S groups may be reduced during glycolysis under both anaerobic and aerobic conditions. The reduction was effected by systems producing DPNH or TPNH or by the addition of DPNH alone; the addition of TPNH was not tried. The effect of the pea-seed extract in reducing the protein S–S groups of the pea-seed glyceraldehyde 3-phosphate dehydrogenase preparation and reactivating the enzyme indicated that this was an enzymic reduction. There was no evidence that cystine reductase or GSSG reductase (which were both present in the pea-seed extract) was involved in this process. Prolonged dialysis, which would lower the concentration of any cysteine or GSH present, did not markedly decrease the rate of reduction of protein S–S groups. Whereas the cystine reductase (Romano & Nickerson, 1954) and the GSSG reductase (Mapson & Goddard, 1951; Conn & Vennesland, 1951) of pea seeds are nucleotide specific, both DPNH and TPNH functioned in reduction of protein S–S groups. The ability of the protein S–S-group-reducing system to utilize both DPNH and TPNH did not appear to be due to pyridine nucleotide transhydrogenase activity.

The present investigation has indicated the probable mechanisms responsible for the reversible aerobic inhibition of glycolysis in pea-seed extracts. It is suggested that glyceraldehyde 3-phosphate dehydrogenase is inhibited by the oxidation of essential SH groups and activated by a DPNH-mediated reduction of the constituent S–S groups (Fig. 4), DPNH being generated through the action of glyceraldehyde 3-phosphate dehydrogenase in

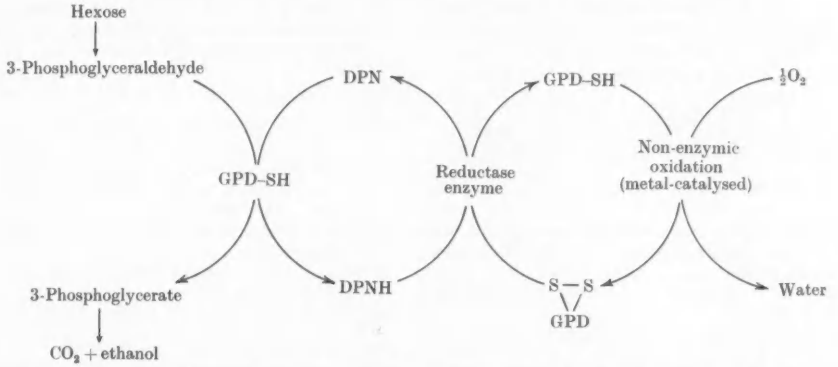

Fig. 4. Suggested mechanism for the reversible aerobic inhibition of glycolysis in the pea-seed extract. GPD, glyceraldehyde 3-phosphate dehydrogenase in the active (SH) and inactive (S–S) forms.

glycolysis. It appears that a balance between these two processes determined the ratio between the SH and S–S groups of glyceraldehyde 3-phosphate dehydrogenase and, through this, the glycolytic activity. It has been calculated (Hatch & Turner, 1959) that not more than 5 % of the DPNH produced during maximum-rate aerobic glycolysis would be necessary to maintain the protein SH-group content at the optimum level for glycolysis. The possible relationship between the aerobic inhibition of glycolysis in the pea-seed extract and the Pasteur effect was discussed previously (Hatch & Turner, 1959).

DPN protects the essential SH groups of mammalian glyceraldehyde 3-phosphate dehydrogenase from oxidation by cystine or GSSG (Rapkine, Rapkine & Trpinac, 1939; Labeyrie, 1949). It was suggested previously that this may explain the effect of DPN concentration on aerobic glycolytic activity in the pea-seed extract (Hatch & Turner, 1959). In the present investigation it was shown that added DPN had little effect on the rate of inactivation of glyceraldehyde 3-phosphate dehydrogenase or the oxidation of protein SH groups in air, although if sufficient DPN was added to aerobic glycolytic digests the concentration of protein SH groups increased. As this effect was probably due to the enzymic reduction of protein S–S groups by DPNH, the rate of reduction may have been controlled by the absolute concentration of DPNH, which at equilibrium may depend upon the level of DPN added. When the DPN concentration was approximately 100 μM or more, glycolytic activity in air was not inhibited (Hatch & Turner, 1959). It appears that at this critical concentration of DPN the reduction of protein S–S groups was more rapid than the oxidation of protein SH groups.

SUMMARY

1. Processes responsible for the oxidation of sulphydryl groups and the reduction of disulphide groups of glyceraldehyde 3-phosphate dehydrogenase in a pea-seed extract were examined.

2. The inhibition of glyceraldehyde 3-phosphate dehydrogenase in aerobic glycolytic digests was due to the oxidation of the essential sulphydryl groups of the enzyme. Evidence indicated that the oxidation of protein sulphydryl groups may have been a non-enzymic process catalysed by traces of heavy metals.

3. Reduction of protein disulphide groups in the pea-seed extract occurred in the presence of reduced diphosphopyridine nucleotide or of systems which generated reduced di- or tri-phosphopyridine nucleotide. Under these conditions glyceraldehyde 3-phosphate dehydrogenase in the extract was activated.

4. In the presence of reduced diphosphopyridine nucleotide and a heat-labile component of the pea-seed extract, enzyme activity and the protein sulphydryl group concentration of a pea-seed glyceraldehyde 3-phosphate dehydrogenase preparation were increased.

5. It is suggested that aerobic glycolytic activity in the pea-seed extract was controlled by processes which mediated the oxidation of sulphydryl groups and the reduction of disulphide groups of glyceraldehyde 3-phosphate dehydrogenase.

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REFERENCES

Balazs, R. & Richter, D. (1958*a*). *Biochem. J.* **68**, 5P.
Balazs, R. & Richter, D. (1958*b*). *Biochem. J.* **69**, 57P.
Benesch, R. & Benesch, R. E. (1945). *Arch. Biochem.* **19**, 35.
Conn, E. E. & Vennesland, B. (1951). *J. biol. Chem.* **192**, 17.
Fromageot, C. (1951). In *The Enzymes*, vol. 2, pt. 1, p. 609. Ed. by Sumner, J. B. & Myrbäck, K. New York: Academic Press Inc.
Hageman, R. H. & Arnon, D. I. (1955). *Arch. Biochem. Biophys.* **55**, 162.
Hatch, M. D. & Turner, J. F. (1958). *Biochem. J.* **69**, 495.
Hatch, M. D. & Turner, J. F. (1959). *Biochem. J.* **72**, 524.
Haugaard, N., Hess, M. E. & Itskovitz, H. (1957). *J. biol. Chem.* **227**, 605.
Labeyrie, F. (1949). *Bull. Soc. Chim. biol., Paris*, **31**, 1624.
MacDonnell, L. R., Silva, R. B. & Feeney, R. E. (1951). *Arch. Biochem. Biophys.* **32**, 288.
Mandels, G. R. (1956). *J. Bact.* **72**, 203.
Mapson, L. W. & Goddard, D. R. (1951). *Biochem. J.* **49**, 592.
Neufeld, H. A., Green, L. F., Latterell, F. M. & Weintraub, R. L. (1958). *J. biol. Chem.* **232**, 1093.
Racker, E. (1955). *J. biol. Chem.* **217**, 855.
Rapkine, L., Rapkine, S. M. & Trpinac, P. (1939). *C.R. Acad. Sci., Paris*, **209**, 253.
Romano, A. H. & Nickerson, W. J. (1954). *J. biol. Chem.* **208**, 409.
Turner, J. F. (1954). *Nature, Lond.*, **174**, 692.
Turner, J. F. (1957). *Biochem. J.* **67**, 450.
Turner, J. F. & Mapson, L. W. (1958). *Nature, Lond.*, **181**, 270.
Velick, S. F. (1953). *J. biol. Chem.* **203**, 563.
Warburg, O. & Christian, W. (1939). *Biochem. Z.* **303**, 40.
Warburg, O. & Christian, W. (1941). *Biochem. Z.* **310**, 384.

Biochem. J. (1960) **75**, 72

Lactic Dehydrogenase and Cytochrome b_2 of Baker's Yeast

THE AMINO ACID COMPOSITION OF THE CRYSTALLINE ENZYME

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Appleby & Morton (1954, 1959*a*, *b*, 1960) have described the purification and crystallization of yeast lactic dehydrogenase, and the enzymic, chemical and physical properties of the crystalline chromoprotein, which was shown to be identical with cytochrome b_2. This paper describes the amino acid composition as determined qualitatively by paper chromatography and quantitatively with ion-exchange-resin columns. An account of this work was given to the Australian Biochemical Society in August, 1957 (Simmonds, Appleby & Morton, 1957*a*, *b*; Morton, 1958).

MATERIALS

Yeast lactic dehydrogenase (*cytochrome* b_2). The crystalline cytochrome was prepared as described by Appleby & Morton (1959*a*). Unless otherwise stated, the material was recrystallized three times from a solution of the combined first crystals from a number of preparations.

Gelatin. The tyrosine-free protein was a gift from Davis Gelatine Co. Ltd., Sydney, N.S.W.

β-Lactoglobulin. Three-times crystallized material was a gift from Dr F. J. R. Hird.

Organic solvents. These were purified for chromatography by distillation at reduced pressure.

EXPERIMENTAL AND RESULTS

Amino acid composition by paper chromatography

Approx. 3 mg. of once-crystallized enzyme was hydrolysed with 0·1 ml. of 5N-HCl in a sealed ampoule at 105° for 20 hr. After removal of HCl, the residue was dissolved in about 70 μl. of propan-2-ol (acidified with HCl). Suitable portions (4–10 μl.) were applied to the appropriate grade of Whatman chromatography paper. The amino acids were separated by two-dimensional chromatography as described by Dent (1947). The ninhydrin-reactive compounds of the enzyme hydrolysate were identified by comparison with similar chromatograms of a reference mixture of known

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amino acids. The results were confirmed with two-dimensional chromatography as described by Boissonnas (1950). The amino acids identified are shown in Table 1.

Attempts to detect tyrosine and histidine with the Pauly reagent (Dent, 1947) on papers developed in collidine–lutidine solvent (Dent, 1947) were unsuccessful. Because of the intense absorption due to nucleotides associated with the crystalline cytochrome (see Appleby & Morton, 1959*b*, 1960), the spectrophotometric method of Beavan & Holiday (1952) for estimation of tyrosine and tryptophan could not be used. Tyrosine was, however, detected by the following procedure. Approx. 2·5 mg. of twice-recrystallized cytochrome b_2 was hydrolysed with 5N-HCl as already described, the acid was removed, the residue was dissolved in water and the whole solution applied as a spot on Whatman no. 4 chromatographic paper. Reference spots of tyrosine and of hydrolysates of β-lactoglobulin and of gelatin were also applied. The chromatogram was developed in butanol–formic acid–water according to Acher & Crocker (1952). The tyrosine was detected by the reaction with α-nitroso-β-naphthol (see Acher & Crocker, 1952). No tyrosine was detected in the gelatin.

Tryptophan. Tryptophan was estimated by the method of Spies & Chambers (1949). β-Lactoglobulin, which contains 2·5% of tryptophan (Spies & Chambers, 1949), was used as a standard. Portions of β-lactoglobulin (1–4 mg.) and of cytochrome b_2 (1·5 mg.) were dissolved in 5·8 ml. of 19N-H_2SO_4 at room temperature (approx. 20°). After 20 min., approx. 18 mg. of *p*-dimethylaminobenzaldehyde was added to each tube and, after about 12 hr. at room temperature, 0·2 ml. of aq. 0·014% $NaNO_2$. After an additional 30 min. at room temperature, E at 590 mμ was determined with a Beckman spectrophotometer (model DU). It was found that the addition of haemin and of riboflavin phosphate to β-lactoglobulin before treatment caused no interference with the estimation of tryptophan. Hence it was concluded that these prosthetic groups (Appleby & Morton, 1954; 1959*b*) did not interfere with the estimation of tryptophan (0·82%; see Table 2) in cytochrome b_2. It is not known whether the deoxyribose polynucleotide component (Morton, 1958; Appleby & Morton, 1960) caused any interference in this estimation.

Amino acid analysis by resin-column chromatography

The cytochrome b_2 was weighed into tubes with B 19 standard ground-glass sockets, 0·5 ml. of constant-boiling HCl was added to each sample, a reflux condenser was fitted and hydrolysis was carried out on an oil bath at 110°. Duplicate samples of approx. 2 mg. each were used for the acidic and neutral amino acids, and of approx. 3·5 mg. each for the basic amino acids. One sample was hydrolysed for 20 hr. and the other for 70 hr. The hydrolysates were dried *in vacuo* over P_2O_5 and KOH at room temperature for about 3 days to remove HCl, and the residues were then dissolved in a minimum of water and transferred quantitatively to the tops of columns of Dowex-50 resin used for analysis.

The amino acids were separated by ion-exchange chromatography (Moore & Stein, 1951) and were estimated by the colorimetric procedure with ninhydrin as described by Moore & Stein (1948). The apparatus used for collecting and dispensing

Table 1. *Amino acids identified in a hydrolysate of cytochrome* b_2 *by separation by paper chromatography*

Once-crystallized cytochrome b_2 (3 mg.) was hydrolysed with 5N-HCl at 105° for 20 hr. in a sealed ampoule. After drying, the residue was dissolved in 70 μl. of propan-2-ol, acidified with HCl and 4–10 μl. portions were applied to filter paper. Chromatographic procedures were carried out according to the authors quoted and as described in the text. For definition of the abbreviations used for amino acids see *Biochem. J.* (1953), **55**, 5.

Reference	Solvent systems used to develop chromatogram: First system	Second system	Amino acids identified
Dent (1947)	Water-saturated phenol	Collidine–lutidine–water	Intense spots: Ala, Asp, Glu, Gly, leucine group,* Val Weaker spots: Arg, Cys,† Lys, Ser, Thr
Boissonnas (1950)	(*a*) *tert.*-butyl alcohol butan-2-one	*tert.*-butyl alcohol methanol–water	Ala, Asp, Gly and Ser,‡ leucine group,* Thr, Val and two spots unidentified
	(*b*) Phenol–water	Propan-2-ol–water	Ala, Arg, Asp, Cys,† leucine group,* Lys
Acher & Crocker (1952)	Butan-1-ol–formic acid–water	—	Tyr

* Leucine, isoleucine and norleucine are not separated by these procedures.
† Cystine was detected as cysteic acid.
‡ Glycine and serine are not separated in this procedure.

the portions of effluent from the columns has been described by Simmonds (1954) and by Simmonds & Wood (1954). Because of the overlap between glutamic acid and proline, and the low colour yield of proline when treated with ninhydrin at pH 5·0, the effluent fractions were divided into two groups at the period when these acids were expected. One group of alternate tubes from the column was treated with ninhydrin at pH 5·0 for estimation of glutamic acid (Moore & Stein, 1948) and the second group was treated with the ninhydrin reagent of Chinard (1952) for estimation of proline.

All of the commonly occurring amino acids of proteins except tryptophan and cystine are determined by these procedures. Tryptophan was therefore estimated separately as already described. As shown in Table 1, cystine was identified in the enzyme hydrolysate by paper chromatography, but methionine was not detected. Neither methionine nor any of its oxidation products was detected in the effluents from the ion-exchange column. Hence it has been assumed that all of the sulphur (0·74%; Appleby & Morton, 1960) of the cytochrome occurs as cysteine and cystine, and the amount (expressed as ½-cystine) was calculated accordingly. Table 2 shows the results for the 20 and 70 hr. hydrolysates, expressed as percentage (by wt.) of the dried material.

For amino acids other than serine there appeared to be little loss during hydrolysis, and the mean of the results for both hydrolysis periods was used as the best estimate (Table 2). Since the decomposition of serine during hydrolysis of proteins with HCl is well established (Rees, 1946; Hirs, 1954; Smith & Stockell, 1954; Smith, Stockell & Kimmel, 1954), the results for serine were linearly extrapolated to zero time to obtain the best estimate, as given in Table 2. Glutamic acid undergoes decomposition to pyrrolidonecarboxylic acid during chromatography on the resin columns and accordingly the correction factor (1·03) of Moore & Stein (1951) was used to obtain the mean value for this amino acid (Table 2).

DISCUSSION

The qualitative (Table 1) and quantitative (Table 2) analyses are in substantial agreement. It is probable that the two unidentified amino acids detected on the chromatogram according to Boissonnas (1950) were histidine and phenylalanine, which were identified by resin-column chromatography (cf. Tables 1 and 2).

Thus methionine is either absent or present in very small amount, and it has been assumed that all of the sulphur of cytochrome b_2 occurs as cystine and cysteine. If present to the extent of 0·2–0·3%, methionine would have been detected by both column and paper chromatography. The loss of lactic-dehydrogenase activity caused by

Table 2. *Amino acid analysis of crystalline cytochrome* b_2 *by chromatography on ion-exchange resin*

Four-times crystallized enzyme was hydrolysed in constant-boiling HCl at 110° for 20 and 70 hr. as indicated. Amino acids of the hydrolysates were separated on a column of Dowex-50 resin and estimated as described in the text, except that cystine was estimated from the total sulphur content of the enzyme and tryptophan was determined according to Spies & Chambers (1949). Results are expressed as percentage (by wt.) of the dried material.

	Amino acid content (%)			
	20 hr. hydrolysate	70 hr. hydrolysate	Mean	S.D.
Alanine	4·42, 4·51	4·36, 4·33	4·40	0·08
Amide	1·59	1·66	1·62	—
Arginine	4·68	4·48	4·58	—
Aspartic acid	9·90, 10·11	9·71, 9·84	9·89	0·17
½-Cystine	—	—	2·77	—
Glutamic acid	10·62, 11·28	10·68, 10·89	10·87*	0·30
Glycine	3·54, 4·01	3·80, 4·15	3·88	0·23
Histidine	1·05	1·35	1·20	—
Isoleucine	4·86	4·49, 4·31	4·55	0·28
Leucine	9·03	8·77, 9·32	9·04	0·26
Lysine	8·46	8·06	8·26	—
Methionine	—	—	<0·3†	—
Phenylalanine	2·98, 2·65	2·73, 3·20	2·89	0·24
Proline	4·72, 5·00	4·48	4·73	0·26
Serine	3·42, 3·29	2·62, 3·34	3·51‡	—
Threonine	3·28, 3·25	3·20, 3·40	3·28	0·08
Tryptophan	—	—	0·82	—
Tyrosine	3·17, 2·92	2·87, 3·62	3·14	0·33
Valine	6·34	6·10, 6·03	6·16	0·16

* Values are corrected for formation of pyrrolidonecarboxylic acid.
† Not detected in column effluent.
‡ Value was obtained by extrapolation of results to zero time of hydrolysis.

oxidizing agents (Appleby & Morton, 1959*b*) and the inhibition by *p*-chloromercuribenzoate (Appleby & Morton, 1954, 1959*b*) indicate that cysteine is present.

Table 3 shows the amino acid nitrogen as a percentage of the total nitrogen, and the residue weight of each amino acid as a percentage of the dry weight of the enzyme. Crystalline cytochrome b_2 contains $15{\cdot}2 \pm 0{\cdot}4\%$ of nitrogen and approx. 14·2% of amino acid nitrogen (Appleby & Morton, 1960). The amino acids (Table 3) represent 84% of the total nitrogen and approx. 91% of the amino acid nitrogen. However, only 74% of the total weight is accounted for by amino acids (Table 3). Crystalline cytochrome b_2 contains approx. 6% of a deoxyribose polynucleotide, approx. 0·8% of protohaem and approx. 0·9% of riboflavin phosphate (Appleby & Morton, 1954, 1959*b*, 1960). Thus these results account for about 81% of the dry weight of the enzyme. The cause of the discrepancy between the recoveries, based on nitrogen and dry-weight estimations, is not known.

Table 3 shows the number of residues of each amino acid/10^4 g. of protein and the number of residues/mole of enzyme-bound haem. Crystalline cytochrome b_2 has a weight of about 80 000 g./mole

Table 3. *Amino acid composition of crystalline cytochrome* b_2 *(yeast lactic dehydrogenase)*

Values are derived from the results given in Table 2.

	Amino acid N as % of total N	Residue wt. as % of dry wt.	Residues/ 10^4 g. of enzyme	Residues/ mole of haem
Alanine	4·55	3·51	4·94	39·5
Amide	9·33	1·62	10·12	81·0
Arginine	9·70	4·11	2·63	21·0
Aspartic acid	6·85	8·55	7·44	59·5
½-Cystine	2·12	2·35	2·31	18·5
Glutamic acid	6·81	9·54	7·39	59·1
Glycine	4·77	2·95	5·17	41·4
Histidine	2·14	1·06	0·77	6·2
Isoleucine	3·20	3·92	3·47	27·8
Leucine	6·35	7·80	6·90	55·2
Lysine	10·42	7·24	5·66	45·7
Methionine*	—	—	0	0
Phenylalanine	1·61	2·57	1·75	14·0
Proline	3·79	3·99	4·11	32·9
Serine	3·08	2·91	3·31	26·5
Threonine	2·52	2·78	2·76	22·1
Tryptophan	0·79	0·80	0·43	3·4
Tyrosine	1·60	2·83	1·73	13·8
Valine	4·85	5·21	5·26	42·1

* Not detected either in column effluent or by paper chromatography (cf. Table 1 and text).

Table 4. *Comparative amino acid compositions of cytochrome* b_2 *and some other proteins*

Amino acid composition is expressed as g. of amino acid/100 g. of protein. Values for cytochrome b_2 have been calculated for the flavohaemoprotein, free of polynucleotide (cf. Appleby & Morton, 1960).

Enzyme	Yeast alcohol dehydrogenase	Pig-heart diaphorase	Yeast cytochrome b_2 (lactic dehydrogenase)	Yeast cytochrome c
Type of protein	Diphosphopyridine nucleotide-linked protein	Flavoprotein	Flavohaemoprotein	Haemoprotein
Reference	Lange (1956)	Rees & Savage (1958)	This paper	Nunnikhoven (1958)
Alanine	6·6	6·9	4·6	4·5
Amide	—	1·5	1·7	1·6
Arginine	4·9	4·5	4·8	3·7
Aspartic acid	8·6	10·0	10·4	11·0
½-Cystine	4·4	—	2·9	1·8
Glutamic acid	6·6	12·3	11·5	10·0
Glycine	5·9	6·4	4·1	5·8
Histidine	2·5	3·3	1·3	4·0
Isoleucine	7·0	6·5	4·8	3·8
Leucine	7·1	8·0	9·5	6·8
Lysine	7·8	9·7	8·7	16·6
Methionine	5·1	2·0	<0·3	2·1
Phenylalanine	5·7	4·4	3·1	4·4
Proline	4·8	4·6	4·9	3·6
Serine	5·8	4·3	3·7	3·3
Threonine	6·6	5·3	3·5	7·2
Tryptophan	1·7	—	0·9	1·6
Tyrosine	6·0	2·9	3·3	5·7
Valine	6·9	7·0	6·5	2·6
Mol. wt. (g.)	150 000*	81 000†	75 000‡	13 200§

* Hayes & Velick (1954). † Savage (1957). ‡ Minimum weight (g.)/mole.
§ Based on dry weight; 11 800 g./mole based on total protein (Nunnikhoven, 1958).

of haem (Appleby & Morton, 1959*b*). Table 3 shows that alanine, aspartic acid, ½-cystine and serine would occur with exactly 0·5 of a residue/mole of haem, but no amino acids would occur with 0·5 of a residue/mole if there are two haem groups/mole of enzyme, as seems likely (Appleby & Morton, 1960).

As yet, cytochrome *c* (de Barbieri & Scevola, 1955; Ehrenberg & Theorell, 1955; Nunnikhoven, 1958; Leaf, Gillies & Pirie, 1958) is the only cytochrome other than cytochrome b_2 for which the amino acid composition has been determined (see Morton, 1958). Table 4 shows the amino acid composition of cytochrome b_2 (yeast lactic dehydrogenase) expressed as g. of amino acid/100 g. of polynucleotide-free enzyme, on which basis this flavohaemoprotein may be directly compared with yeast alcohol dehydrogenase, yeast cytochrome *c* and pig-heart diaphorase (Table 4). These represent a typical diphosphopyridine nucleotide-linked dehydrogenase, a haemoprotein and a flavoprotein respectively. All are directly concerned in hydrogen (or electron) transport. Table 4 shows that the flavohaemoprotein (cytochrome b_2) shows a general similarity in composition to each of the other proteins and especially to diaphorase, the only flavoprotein for which the amino acid composition is available. The comparative amino acid composition of cytochrome b_2 and cytochrome *c* has been discussed elsewhere by Morton (1958). Whereas the amino acid compositions of the proteins shown in Table 4 are rather similar, they differ from the compositions of other enzymes such as catalase, pepsin and α-amylase (see Dixon & Webb, 1958). It is possible therefore that certain features of the amino acid patterns are related to their enzymic functions.

Cytochrome b_2 thus shows no unique features of amino acid composition, except for the apparent absence (less than 0·3%) of methionine. Most proteins and almost all enzymes so far studied contain between 0·5 and 5·0% of methionine. The apparent absence of methionine in cytochrome b_2 supports the conclusion based on other studies (Appleby & Morton, 1954, 1959*a*, *b*, 1960) that the crystalline material contains only one protein.

SUMMARY

1. Crystalline cytochrome b_2 was hydrolysed with 5N-hydrochloric acid at 105° for 20 hr. and the liberated amino acids were separated by paper chromatography according to Dent (1947) and Boissonnas (1950). Most of the commonly occurring amino acids, except methionine, were detected.

2. The amino acids in hydrolysates obtained after 20 and 70 hr. at 110° with constant-boiling hydrogen chloride solution were separated on a column of Dowex-50 according to Moore & Stein (1951) and estimated by the colour developed with ninhydrin. Methionine was not detected.

3. Comparison of the amino acid composition of cytochrome b_2, yeast cytochrome *c*, yeast alcohol dehydrogenase and pig-heart diaphorase shows considerable similarity between these several proteins.

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REFERENCES

Acher, R. & Crocker, C. (1952). *Biochim. biophys. Acta*, **9**, 704.
Appleby, C. A. & Morton, R. K. (1954). *Nature, Lond.*, **173**, 749.
Appleby, C. A. & Morton, R. K. (1959*a*). *Biochem. J.* **71**, 492.
Appleby, C. A. & Morton, R. K. (1959*b*). *Biochem. J.* **73**, 539.
Appleby, C. A. & Morton, R. K. (1960). *Biochem. J.* (in the Press).
Beavan, G. H. & Holiday, E. R. (1952). *Advanc. Protein Chem.* **7**, 319.
Boissonnas, R. A. (1950). *Helv. chim. acta*, **33**, 1966.
Chinard, F. P. (1952). *J. biol. Chem.* **199**, 91.
de Barbieri, A. & Scevola, M. E. (1955). *Bull. Soc. ital. Biol. sper.* **31**, 688.
Dent, C. E. (1947). *Biochem. J.* **41**, 240.
Dixon, M. & Webb, E. C. (1958). *Enzymes*, p. 483. London: Longmans, Green and Co.
Ehrenberg, A. & Theorell, H. (1955). *Acta chem. scand.* **9**, 1193.
Hayes, J. E. & Velick, S. F. (1954). *J. biol. Chem.* **207**, 225.
Hirs, C. H. W. (1954). *Fed. Proc.* **13**, 230.
Lange, K. (1956). *Hoppe-Seyl. Z.* **303**, 272.
Leaf, G., Gillies, N. E. & Pirie, R. (1958). *Biochem. J.* **69**, 605.
Moore, S. & Stein, W. H. (1948). *J. biol. Chem.* **176**, 367.
Moore, S. & Stein, W. H. (1951). *J. biol. Chem.* **192**, 663.
Morton, R. K. (1958). *Rev. pure appl. Chem.* **8**, 161.
Nunnikhoven, R. (1958). *Biochim. biophys. Acta*, **28**, 108.
Rees, M. W. (1946). *Biochem. J.* **40**, 632.
Rees, M. W. & Savage, N. (1958). Cited as personal communication by Dixon, M. & Webb, E. C. (1958). *Enzymes*. London: Longmans, Green and Co.
Savage, N. (1957). *Biochem. J.* **67**, 146.
Simmonds, D. H. (1954). *Aust. J. biol. Sci.* **7**, 98.
Simmonds, D. H., Appleby, C. A. & Morton, R. K. (1957*a*). *Aust. J. Sci.* **20**, 59.
Simmonds, D. H., Appleby, C. A. & Morton, R. K. (1957*b*). Appendix I in Appleby, C. A. Ph.D. Thesis: University of Melbourne.
Simmonds, D. H. & Wood, K. I. (1954). *Analyt. Chem.* **26**, 1850.
Smith, E. L. & Stockell, A. (1954). *J. biol. Chem.* **207**, 501.
Smith, E. L., Stockell, A. & Kimmel, J. R. (1954). *J. biol. Chem.* **207**, 551.
Spies, J. R. & Chambers, D. C. (1949). *Analyt. Chem.* **21**, 1249.

The Potassium and Sodium Contents of Sheep Tissues in Relation to the Potassium Content of the Erythrocytes and the Age of the Animal

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Abderhalden (1898) and Kerr (1937) showed that the concentrations of electrolytes in the erythrocytes of different mammalian species varied considerably. Kerr (1937) observed that a wide range occurred in the sodium and potassium concentrations of the erythrocytes of sheep and Widdas (1954) and Evans (1954) showed that the concentrations varied around either of two means. These two types of sheep have been named HK (high potassium: approximately 83 m-equiv. of potassium/l. of erythrocytes) and LK (low potassium: approximately 23 m-equiv. of potassium/l. of erythrocytes). It is also possible to recognize intermediate types (Evans, 1957*a*) but these are rare in British breeds. The HK and LK characters are heritable (Evans & King, 1955; Evans, King, Cohen, Harris & Warren, 1956), and it is possible that the two types may differ in other physiological characters as their frequency varies from breed to breed (Evans & Mounib, 1957; Evans, Harris & Warren, 1958*a*) and their geographic distribution is not a random one (Evans, Harris & Warren, 1958*b*).

The object of the work reported here was to see if comparable differences could be found in other tissues of the sheep. The erythrocytes of the rat and the cat contain 100 and 6 m-equiv. of potassium/l. of erythrocytes respectively, and these can therefore be considered as HK and LK animals respectively. The data of Harrison & Darrow (1938) and Yannet & Darrow (1938) suggest that a significant difference also exists between the potassium and sodium concentrations of tissues such as the skeletal muscle and liver of these animals.

Sheep of different ages had to be used in the experiments and as the age of the animal, irrespective of the blood type, influenced the results, further attention was given to this factor.

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MATERIAL AND METHODS

General

The tissues of sheep from three age groups were used, namely lambs approx. 1 year old (88 animals); adult sheep approx. 4 years old (33 animals) and aged sheep approx. 6–8 years old (30 animals). Most specimens were obtained from the local abattoir but the 10 aged ewes which were used to obtain a number of different tissues were maintained on a constant ration for at least 2 weeks before slaughter. The sheep used were Scottish Blackface or crosses with that breed, or Border Leicester ewes (six animals only).

The animals were shot with a captive-bolt humane killer and then were bled. A blood sample was taken at this time. Tissues were collected within 3 min. after slaughter. The amount of blood which remains in tissues is relatively small if the animal is allowed to bleed freely before tissue samples are taken (Fenn, 1939).

The investigation was carried out in two stages: (*a*) The potassium and sodium concentrations in kidney, heart and liver tissue were determined in three age groups of sheep. All age groups included HK and LK animals. (*b*) Ten aged Scottish Blackface sheep (five HK and five LK) were used to provide a wider range of tissues and a more detailed study of them in relation to the erythrocyte type of the animals. Skeletal muscle, liver, spleen, kidney, heart, brain, duodenum, rumen and rumen epithelium were examined for their potassium and sodium concentrations as well as for their water and fat contents. The blood content was determined for all tissues except the brain, duodenum, rumen and rumen epithelium, as Laramore & Grollman (1950) found it to be of negligible importance in these tissues after exsanguination.

Tissues were taken from the same part of each organ each time. Skeletal muscle: biceps femoris. Liver: peripheral tissue devoid of large ducts or vessels. Spleen: the capsule was removed and tissue which did not contain large vessels was selected. Kidney: the cortex of the left kidney; Eichelberger & Bibler (1940) found identical potassium and sodium values for the right and left kidneys. Heart: Davies, Davies, Francis & Whittam (1952) reported that potassium and sodium concentrations vary from one part of the heart to another; heart muscle was taken from a point approx. 5 mm. to the right of the apex, where the ventricular septum and the wall of the left ventricle meet (endo- and epi-cardial tissue was removed). Brain: cortical and medullary tissue in approximately equal quantities were taken from the anterior part of the dorsal aspect of

the cerebrum adjacent to the longitudinal fissure. Small intestine: a section through the whole wall of the organ was taken approx. 45 cm. caudal to the opening of the common bile duct. Rumen and rumen epithelium: samples were taken from the anterior part of the dorsal sac of the rumen about 12 cm. from the point of entry of the oesophagus; the epithelium is easily stripped.

All tissues were quickly blotted on ashless filter paper and visible fat was removed. Tissues from the digestive tract received a quick wash in water to remove adherent digesta. Duplicate or triplicate samples of each tissue were analysed.

Chemical methods

Potassium and sodium. The method described by Mounib & Evans (1957) was used in which tissues were extracted with 2% (w/v) trichloroacetic acid for 24 hr. in the cold.

Water. Tissues were weighed, frozen at $-20°$ and then dried over P_2O_5 under vacuum to constant weight.

Fat. This was determined on dry tissues by the method of Hastings & Eichelberger (1937).

Residual blood in tissues. The benzidine method of Bing & Baker (1931) as modified by Laramore & Grollman (1950) was used.

Statistical analysis. When the numbers of animals in the subclasses were not equal, the analysis of variance was made by the method for disproportionate subclass numbers as suggested by Snedecor (1955).

RESULTS

Effect of blood type and age on the concentrations of potassium and sodium in the kidney, heart and liver

The concentrations of potassium and sodium in the kidney, heart and liver of lambs, adult and aged sheep of both blood types are given in Table 1. The great majority of these samples were obtained from the abattoir and it was not possible therefore to obtain equal numbers of HK and LK animals. Cross-bred sheep were most commonly available and therefore approximately three times as many LK as HK sheep were sampled for kidney and heart tissue and approximately twice as many for liver tissue. As the total number of animals sampled was large the unequal distribution of the two types does not seriously detract from the results.

The LK sheep had a significantly higher concentration of sodium in the kidney than did HK sheep ($P < 1{\cdot}0\%$). No other significant difference with respect to blood type and tissue concentrations was found. Age and blood type were significantly related for the potassium concentrations in the kidney ($P < 5\%$). The effect of age was highly significant with respect to the potassium ($P < 1{\cdot}0\%$) and sodium ($P < 0{\cdot}1\%$) concentrations of the kidney. The older sheep had lower concentrations of potassium and higher concentrations of sodium. The potassium concentration of the heart also decreased significantly with age ($P < 0{\cdot}1\%$). The HK animals had a higher potassium to sodium ratio in all three tissues but the difference was significant only for the kidney ($P < 5\%$). There was a tendency for the ratio to decrease with advancing age (Table 2). The effect of age on the ratio was highly significant in kidney ($P < 0{\cdot}1\%$) and heart tissue ($P < 1{\cdot}0\%$).

Table 1. *Concentrations of potassium and sodium in kidney, heart and liver tissue of LK and HK sheep of different ages*

Differences between LK and HK sheep (irrespective of age) are not significant except for the sodium concentrations in the kidney ($P < 1{\cdot}0\%$). Effects of age (irrespective of the potassium type of the animal) are significant for the potassium ($P < 1{\cdot}0\%$) and sodium ($P < 0{\cdot}1\%$) concentrations of kidney and for the potassium concentrations of the heart muscle ($P < 0{\cdot}1\%$). Interaction between type and age is significant only for the potassium concentration in the kidney. The numbers of animals examined in each class are shown in Table 2.

Age group	Blood type	Potassium (m-equiv./kg. wet wt.; mean ± S.E.)			Sodium (m-equiv./kg. wet wt.; mean ± S.E.)		
		Kidney	Heart	Liver	Kidney	Heart	Liver
Lambs	LK	71·54±0·43	83·98±0·47	81·45±0·80	66·20±0·58	38·84±0·30	33·30±1·92
	HK	73·66±1·32	85·92±1·01	85·71±3·24	63·76±1·06	37·96±0·78	32·95±2·58
	Total	71·91±0·43	84·23±0·43***	83·15±1·53	65·77±0·52**	38·73±0·28	33·16±1·33
Adults	LK	73·08±1·81	80·25±0·79	79·11±0·63	71·59±2·42	38·63±0·68	34·58±0·90
	HK	68·84±0·90	78·22±1·25	78·02±1·28	66·07±1·43	37·33±0·61	34·59±1·16
	Total	71·54±1·32	79·76±0·68	78·85±0·56	69·59±1·90	38·32±0·55	34·58±1·05
Aged	LK	67·08±1·93	77·98±0·98	79·47±0·90	72·57±1·69	38·89±0·69	36·51±0·91
	HK	68·73±1·14	79·39±0·74	81·03±1·34	69·04±2·15	38·91±1·07	34·20±0·99
	Total	67·91±1·09*	78·59±0·64	80·14±0·77	70·82±1·42	38·90±0·59	35·51±0·69

* Differences between the potassium contents of the kidneys of aged sheep and those of adult sheep are on the borderline of significance at the 5% level; differences between aged sheep and lambs are highly significant ($P < 0{\cdot}1\%$).

** Differences between the sodium concentrations of the kidney tissue of lambs and those of aged sheep are highly significant ($P < 1\%$).

*** Differences between the potassium concentrations of heart muscle of lambs and those of both adult and aged sheep are highly significant ($P < 0{\cdot}1\%$).

Table 2. *Ratio of potassium to sodium in kidney, heart and liver tissue in HK and LK sheep of different ages*

Effect of blood type (irrespective of age) is significant only in the kidney ($P < 5{\cdot}0\%$). Effect of age (irrespective of blood type) is highly significant in the kidney ($P < 0{\cdot}1\%$) and heart ($P < 1{\cdot}0\%$).

Age group	LK sheep		HK sheep		Total	
	No. of animals	Mean ratio ± S.E.	No. of animals	Mean ratio ± S.E.	No. of animals	Mean ratio ± S.E.
		Kidney				
Lambs	47	1·09 ± 0·01*	10	1·16 ± 0·03	57	1·10 ± 0·01
Adult	7	1·03 ± 0·05	4	1·04 ± 0·04	11	1·04 ± 0·04
Aged	5	0·93 ± 0·05	5	1·00 ± 0·03	10	0·97 ± 0·03**
		Heart				
Lambs	48	2·17 ± 0·01	7	2·27 ± 0·06	55	2·18 ± 0·02***
Adult	16	2·09 ± 0·05	5	2·10 ± 0·06	21	2·09 ± 0·04
Aged	17	2·02 ± 0·06	13	2·06 ± 0·07	30	2·04 ± 0·05
		Liver				
Lambs	3	2·47 ± 0·16	2	2·63 ± 0·30	5	2·53 ± 0·13
Adult	16	2·31 ± 0·07	5	2·34 ± 0·21	21	2·32 ± 0·02
Aged	17	2·20 ± 0·07	13	2·40 ± 0·10	30	2·29 ± 0·06

* Differences between LK and HK lambs are significant ($P < 5{\cdot}0\%$).
** Differences between aged sheep and lambs are highly significant ($P < 0{\cdot}1\%$).
*** Differences between lambs and adult sheep are significant ($P < 5{\cdot}0\%$) and those between lambs and aged sheep are highly significant ($P < 1{\cdot}0\%$).

A study of the concentrations of potassium and sodium in individual samples of all three tissues showed that they were inversely related in spite of a considerable scatter. Regression equations indicate that a negative correlation existed, varying from $-0{\cdot}36$ to $-0{\cdot}56$ (P 0·1–2·0%).

The concentrations of potassium in the heart and in the liver from the same adult or aged sheep were positively related (r 0·41 and $P < 1\%$); that is, they moved in the same direction.

Potassium and sodium concentrations of the tissues of aged sheep corrected for fat content or blood content or both

Five HK and five LK aged Scottish Blackface ewes were used. The quantities of fat, water and trapped blood were determined in nine tissues. The fat content of the skeletal muscle was significantly higher in LK than in HK sheep ($P < 5\%$). There were no other significant differences between HK and LK sheep in relation to fat content, water content or trapped blood. Because the full nutritional history of these ewes was not known and because of the importance of skeletal-muscle fat content in animal husbandry a further comparison of the fat content of muscle was made between two groups of HK and LK lambs of the same breed. In this experiment no difference was found.

No significant differences were found between the potassium and sodium concentrations or the potassium to sodium ratios in any tissue other than rumen epithelium (values for HK animals were higher than those for LK animals: $P < 1{\cdot}0\%$) although there was a tendency (approaching significance) in four of the tissues examined (skeletal muscle, liver, spleen and kidney) for the LK sheep to have lower potassium concentrations, higher sodium concentrations and lower potassium to sodium ratios than the HK sheep (Table 3). In each of the nine tissues examined there was a tendency for an inverse relationship to exist between the potassium and sodium concentrations, in agreement with the previous results.

DISCUSSION

Suitable techniques for obtaining tissues from experimental animals without any transfer of fluids from one compartment to another are difficult, and although exsanguination is known to cause a movement of interstitial fluid into the vascular system many previous investigations on the potassium and sodium contents of tissues have been made after the animal has been so treated. It is also likely that a movement of fluid from the intracellular to the extracellular phase will take place but this latter movement will probably be confined, as far as electrolytes are concerned, to ions not electrostatically held by non-diffusible material such as proteins. It is impossible to say how far the results presented here have been influenced by such movements but as all animals were treated in the same way any bias introduced will be in the same direction.

Table 3. *Concentrations of potassium and sodium, the ratio of potassium to sodium and the percentage of blood, fat and water in different tissues of the sheep*

(1), Estimations are for fat-free, blood-free, wet tissue; (2), estimations are not corrected for fat or blood in wet tissue; (3), estimations are for fat-free wet tissue.

		Potassium, sodium and ratio (m-equiv./kg. of wet tissue)					
		LK (five animals)			HK (five animals)		
Tissue	Calculation	K	Na	K/Na	K	Na	K/Na
Skeletal muscle	(1)	108·8±1·73	21·6±0·93	5·00±0·319	110·4±1·67	19·4±0·80	5·71±0·213
Liver	(1)	86·7±1·66	36·0±1·67	2·43±0·114	88·6±1·66	31·8±1·15	2·80±0·114
Spleen	(1)	111·0±1·60	34·0±1·00	3·28±0·120	115·1±2·55	31·0±0·90	3·72±0·155
Kidney	(1)	68·8±2·04	73·0±1·60	0·95±0·049	70·1±1·19	69·6±2·30	1·01±0·036
Heart	(1)	83·4±1·82	35·6±1·16	2·36±0·121	82·7±1·15	36·4±1·37	2·28±0·105
Brain	(2)	89·6±1·26	53·8±1·30	1·67±0·035	88·7±1·40	53·6±1·23	1·66±0·059
Small intestine	(3)	70·9±1·44	51·5±2·42	1·39±0·070	70·3±2·18	52·8±2·66	1·35±0·031
Rumen	(3)	68·2±1·43	55·3±1·53	1·24±0·076	69·3±2·25	57·1±2·33	1·23±0·077
Rumen epithelium	(3)	42·6±1·55	60·2±2·71	0·72±0·054	51·8±0·98**	64·5±1·93	0·81±0·048

	Percentage of blood in wet tissue (ten animals)	Fat and water (g./kg. wet wt.)			
		LK (five animals)		HK (five animals)	
		Fat	Water	Fat	Water
Skeletal muscle	1·12	44·0±5·8*	779·9±5·8 (1)	26·9±4·0*	779·4±4·7 (1)
Liver	1·46	33·0±4·4	721·8±4·6 (1)	33·6±2·4	717·6±4·9 (1)
Spleen	5·34	15·4±1·2	788·8±3·0 (1)	17·4±0·1	787·7±2·5 (1)
Kidney	1·29	14·9±2·3	806·0±4·0 (1)	12·5±1·6	805·3±2·6 (1)
Heart	1·97	14·4±2·9	793·0±2·2 (1)	16·5±1·6	788·5±1·8 (1)
Brain	—	89·4±10·0	808·3±16·0 (2)	110·2±16·5	810·8±9·3 (2)
Small intestine	—	12·2±1·7	828·3±3·8 (3)	10·4±2·3	835·5±3·5 (3)
Rumen	—	13·8±2·9	812·7±3·4 (3)	10·4±1·9	814·8±3·3 (3)
Rumen epithelium	—	5·8±0·4	808·9±4·8 (3)	7·5±1·5	808·3±0·9 (3)

* Differences in fat content of skeletal muscle between LK and HK sheep are significant ($P < 5\%$).
** Differences in potassium concentration of rumen epithelium between LK and HK animals are highly significant ($P < 1{\cdot}0\%$).

The concentrations of sodium and potassium in the plasma are the same for HK and LK sheep and show little variation, so that a similar constancy can be expected in the interstitial fluid. For this reason it is unlikely that the residual interstitial fluid present in the tissues examined will have introduced a serious error in the comparisons made unless there was great variation in the quantities present.

The results which were obtained show that with the exception of the kidney and possibly the rumen epithelium (two organs in which active transport of sodium is known to occur) there are no marked differences between HK and LK sheep in the potassium and sodium contents of the tissues examined so that the difference in the erythrocytes is a somewhat unique feature. The meaning of the differences in sodium concentration and ratio of potassium to sodium in the kidney between the HK and LK animals requires closer investigation, as they may be correlated with the differences in water metabolism between these two types of sheep, which was observed by Evans (1957*b*). He noted a tendency for LK sheep to drink less water and excrete less urine than HK sheep. The differences between the two types of sheep in the potassium concentration of the rumen epithelium are interesting, although the effect of washing this tissue must be taken into consideration. The food which was eaten by these sheep was the same and R. N. B. Kay (personal communication) has not found any difference in the potassium or sodium concentrations in the parotid-gland saliva from HK and LK sheep, so it seems unlikely that external factors such as these could influence the results. However, the high concentration of sodium and the low concentration of potassium in the extracellular fluid compared with the intracellular fluid could result in the differences which were observed in the kidney and rumen epithelium if there is a difference in the proportion of intracellular space between HK and LK animals in these tissues.

Harris, McDonald & Williams (1952), working with anaesthetized sheep, found relatively low values for potassium in skeletal muscle: range 75–85 m-equiv./kg. wet wt. (compared with 109·6 m-equiv./kg. in the present investigation). As Harris *et al.* (1952) were working in Australia and give potassium concentrations for erythrocytes of the same order as those found by Denton, Wynn, McDonald & Simon (1951) and Evans (1957*a*) in merino sheep it can be assumed that their data for skeletal muscle refer to merino sheep. The difference between the data for skeletal muscle in this paper and those of Harris *et al.* (1952) is of interest in that the merino sheep has a mean erythrocyte-potassium concentration for LK sheep of approximately 10 m-equiv./l., whereas the mean value for LK British Hill breeds such as the Scottish Blackface is approximately 23 m-equiv./l.

The effects of age on the kidney are similar to those observed by Lowry, Hastings, McCay & Brown (1946) in rats.

The negative correlation between the concentrations of potassium and sodium that was found to exist in the kidney, heart and liver tissue in the first experiment and the confirmatory tendency for such a relationship to exist in the nine tissues subsequently studied in the second experiment may mean that the extra- and intra-cellular phases varied from one animal to another for any one tissue, and that an increase in the extracellular phase (indicated by the increase in the concentration of sodium) is met by a decrease in the intracellular phase (indicated by the decrease in the concentration of potassium). The observation that the concentrations of potassium in the heart and liver are correlated may mean that an increase in the intracellular phase of the liver is associated with an increase in the intracellular phase of the heart in the same animal. On the other hand, this may indicate that individuals have intra- and extra-cellular phases which are specific to themselves and which may be distinctly different from those of other members of the same group or breed. Fowler & Upfill (1955) suggested that the differences in the extracellular phase, estimated as sodium thiosulphate space, which they found between individual rabbits were real differences and specific to individuals. Repeated measurements on one particular tissue (e.g. skeletal muscle) of the same animal will prove or reject this hypothesis. This could be done by the well-established technique of skeletal-muscle biopsy.

SUMMARY

1. Potassium and sodium concentrations in kidney, heart and liver tissue in sheep with either high (HK) or low (LK) concentrations of potassium in their erythrocytes were determined in young, adult and aged individuals.

2. The concentration of sodium in the cortex of the kidneys of LK animals was significantly higher (about 5·6 %) than in those of HK animals. Differences of the order of those found in the erythrocytes of sheep were, however, not found in any tissue.

3. The concentration of potassium decreased with age in both the kidney and the heart but not in the liver; the average difference between young and aged sheep was 5·7 % in the kidney and 6·9 % in the heart. A reciprocal rise in sodium occurred in the kidney and the average difference between young and aged sheep was 7·4 %.

4. The ratio of potassium to sodium decreased significantly with advancing age in kidney and heart tissue.

5. Nine tissues were analysed from a further five LK and five HK aged ewes. The skeletal muscle, liver, spleen and kidney (expressed on a fat- and blood-free basis) of the aged HK ewes tended to have higher concentrations of potassium (1·5, 2·2, 3·6 and 1·9% respectively) and lower concentrations of sodium (10·7, 12·3, 9·2 and 4·8% respectively) than those of the aged LK ewes.

6. Rumen epithelium from LK sheep had a significantly lower potassium concentration (19·5%) than that from HK animals.

7. The fat content of the skeletal muscle of aged LK sheep was significantly higher (17 g. of fat/kg. wet wt. or about 48%) than that of aged HK sheep but this difference was not found in a group of lambs of the same breed.

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REFERENCES

Abderhalden, E. (1898). *Hoppe-Seyl. Z.* **25**, 65.
Bing, F. C. & Baker, R. W. (1931). *J. biol. Chem.* **92**, 589.
Davies, F., Davies, R. E., Francis, E. T. B. & Whittam, R. (1952). *J. Physiol.* **118**, 276.
Denton, D. A., Wynn, V., McDonald, I. R. & Simon, S. (1951). *Acta med. scand.* **140**, Suppl. 261.
Eichelberger, L. & Bibler, W. G. (1940). *J. biol. Chem.* **132**, 645.
Evans, J. V. (1954). *Nature, Lond.*, **174**, 931.
Evans, J. V. (1957*a*). *J. Physiol.* **136**, 41.
Evans, J. V. (1957*b*). *Nature, Lond.*, **180**, 756.
Evans, J. V., Harris, H. & Warren, F. L. (1958*a*). *Proc. Roy. Soc.* B, **148**, 249.
Evans, J. V., Harris, H. & Warren, F. L. (1958*b*). *Nature, Lond.*, **182**, 320.
Evans, J. V. & King, J. W. B. (1955). *Nature, Lond.*, **176**, 171.
Evans, J. V., King, J. W. B., Cohen, B. L., Harris, H. & Warren, F. L. (1956). *Nature, Lond.*, **178**, 849.
Evans, J. V. & Mounib, M. S. (1957). *J. agric. Sci.* **48**, 433.
Fenn, W. O. (1939). *J. biol. Chem.* **128**, 297.
Fowler, R., jun. & Upfill, J. (1955). *Aust. J. exp. Biol. med. Sci.* **33**, 39.
Harris, H., McDonald, I. R. & Williams, W. (1952). *Aust. J. exp. Biol. med. Sci.* **30**, 33.
Harrison, H. E. & Darrow, D. C. (1938). *J. clin. Invest.* **17**, 77.
Hastings, A. B. & Eichelberger, L. (1937). *J. biol. Chem.* **117**, 73.
Kerr, S. E. (1937). *J. biol. Chem.* **117**, 227.
Laramore, D. C. & Grollman, A. (1950). *Amer. J. Physiol.* **161**, 278.
Lowry, O. H., Hastings, A. B., McCay, C. M. & Brown, A. N. (1946). *J. Geront.* **1**, 345.
Mounib, M. S. & Evans, J. V. (1957). *Analyst*, **82**, 522.
Snedecor, G. W. (1955). *Statistical Methods*, 4th ed. Ames, Iowa: The Iowa State College Press.
Widdas, W. F. (1954). *J. Physiol.* **125**, 18*P*.
Yannet, H. & Darrow, D. C. (1938). *J. clin. Invest.* **17**, 87.

Biochem. J. (1960) **75**, 82

Comparative Biochemistry of Urea Synthesis

3. ACTIVITIES OF UREA-CYCLE ENZYMES IN VARIOUS HIGHER AND LOWER VERTEBRATES*

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In the first paper of this series we reported on a systematic scheme of analysis for determining quantitatively the activity of urea-cycle enzymes in liver extracts of ureotelic animals (Brown & Cohen, 1959*a*). Application of this method to livers of *Rana catesbeiana* tadpoles revealed the induction of the cycle at the onset of metamorphosis (Brown & Cohen, 1958, 1959*b*). It was also found that the activity of the urea-cycle enzymes could account for the rate of urea synthesis by anuran liver as judged by the rate of urea excretion by intact, metamorphosing tadpoles. This offered final and convincing evidence that the urea cycle as formulated in Fig. 1 (see Brown & Cohen, 1958; Cohen & Brown, 1959; Ratner, 1954, 1955) represents the sequence of reactions for the biosynthesis of urea by a ureotelic organism. Moreover, the favourable free-energy changes associated with the

* Part 2: Brown & Cohen (1959*b*).

individual steps of the cycle demonstrate the thermodynamic feasibility of these reactions (Cohen & Brown, 1959).

The acquisition by tadpoles of the biochemical system for the synthesis of urea is thought to mimic in some degree the evolutionary course of nitrogen-excretion mechanisms (Brown & Cohen, 1959*b*). The present paper is devoted to a further study of the evolutionary significance of the urea cycle, and an attempt is made to locate sites of enzymic alterations in the vertebrate evolutionary tree.

METHODS AND MATERIALS

The animals used in the present study were from a variety of commercial and private sources. Fresh livers were used. The preparation of liver extracts by the use of the detergent cetyltrimethylammonium bromide (Technical Grade, Eastman Kodak Co.) and the procedures for the enzymic assays have been described in detail previously (Brown & Cohen, 1959*a*). With shark liver, the usual assay procedures were modified because of the large amount of urea present in whole extracts of liver. (The liver of one shark contained approx. 1% of urea.) Urea yields colour with the diacetyl monoxime reagent used for determining citrulline, the final product in the carbamyl phosphate synthetase and ornithine-transcarbamylase assays. The modification consisted of the addition of urease to the incubation medium to remove the interfering urea. A shark-liver pellet was prepared by homogenizing fresh liver in the cold with 10 vol. of iso-osmotic KCl, centrifuging at 4000 *g* for 30 min. (after an initial centrifuging at 600 *g* to remove nuclei and unbroken cells), washing the pellet twice with iso-osmotic KCl and extracting the pellet with water. Assays were performed on this water extract. Homogenates of lizard and snake liver for arginase assays were prepared in double-distilled water. Cetyltrimethylammonium bromide extracts of such livers also exhibited arginase activity. Urease was Sigma type V (Sigma Chemical Co., St Louis, Mo.).

All assays were performed at 38°. One unit of enzyme equals 1 μmole of product formed/hr. The value, units/g., is based on wet weight of tissue.

The amount of protein (enzyme) employed in the carbamyl phosphate synthetase reaction must be controlled carefully to achieve maximum rates under assay conditions. The course of the reaction for carbamyl phosphate synthetase of an *Amphiuma* liver as a function of protein concentration is depicted in Fig. 2*A*. The rate falls off slightly as the protein concentration is increased. A plot of units/ml. of extract against ml. of extract, as shown in Fig. 2*B*, affords a linear extrapolation to the value on the ordinate axis of units of enzyme/ml. of extract for infinitesimally small protein concentrations. Linearity of carbamyl phosphate synthetase activity with increasing protein concentration can be obtained only when the reaction has proceeded to a small extent, such as is obtainable at low protein (enzyme) concentrations. Whenever possible it is advisable to compare an extrapolated value as indicated with the value obtained in routine assays. Such a procedure permits selection of an enzyme concentration low enough to afford a linear rate during the assay interval. Only then can valid comparisons be made among enzymes prepared from the livers of different species.

RESULTS AND DISCUSSION

The activities of urea-cycle enzymes in the livers of various vertebrates are tabulated in Table 1. The data were obtained during the last 2 years and values reported for the various enzymic activities for a given species are not necessarily simultaneous assays for the several enzymes from given specimens of liver. For purposes of comparison with a common laboratory animal, we have included data of simultaneous assays for all the enzymes (except the cleavage enzyme) from five rat livers (Holtzman albino strain) performed in conjunction with studies on regenerating liver (Kim, 1959).

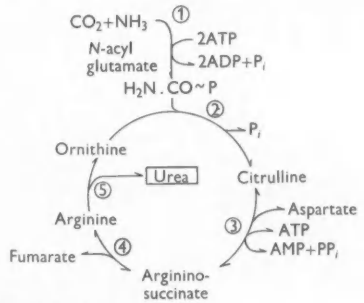

Fig. 1. Krebs & Henseleit (1932) urea cycle as modified and completed by later enzymic studies. ATP, ADP, AMP, Adenosine tri-, di- and mono-phosphate respectively.

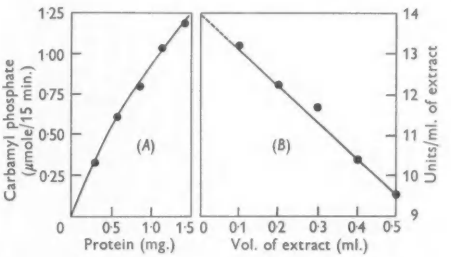

Fig. 2. Carbamyl phosphate synthetase of *Amphiuma means*. *A*, Amount (μmoles) of carbamyl phosphate and protein concentration. *B*, Activity of enzyme (units/ml. of extract) and volume of extract; the straight-line plot extrapolates to 14 units/ml., corresponding to 252 units/g. of liver wet wt.

Table 1. *Activities of urea-cycle enzymes in livers of various species*

Abbreviations and symbols: units/g., μmoles of product formed/hr./g. wet wt. of liver; +, present by qualitative test; −, no assay performed; BLD, assay was performed but activity was not demonstrated (below limit of detection); figures in parentheses indicate number of animals (all other determinations are on single specimens); ±, mean deviation from mean value. For further details see text.

		Carbamyl phosphate synthetase		Ornithine transcarbamylase	
Common name	Binominal name	Units/g.	Units/mg. of protein	Units/g.	Units/mg. of protein
Mudpuppy	*Necturus maculosus maculosus* Rafinesque	16·2±3·6 (3)	0·38±0·09 (3)	2 100±1 000 (3)	51±25 (3)
Salamander	*Ambystoma tigrinum*	360±80 (7)	7·02±0·91 (7)	+	−
Newt	Not identified	378	11·6	−	−
Congo eel	*Amphiuma means*	206±36 (2)	4·70	10 200±2 500 (2)	252
Tadpole	*Rana catesbeiana**				
	Stage VIII	−	−	−	−
	Stages III–X	−	−	−	−
	Paddle stages	−	−	−	−
	Stage XVIII	169	3·77	−	−
	Stage XIX	174	3·93	−	−
	Stages XI–XIX	−	−	−	−
	Stage XXV	−	−	−	−
Bullfrog	*Rana catesbeiana*	980±120 (3)	15·3±5·4 (3)	9 700±2 300 (2)	53·5±28·4 (2)
Leopard frog	*Rana pipiens*	+	−	−	−
Painted turtle	*Chrysemys picta*	644	−	12 800	−
Fowler's toad	*Bufo fowleri*	772	−	16 000	−
Giant toad	*Bufo marinus*	804±45 (3)	11·6±1·2 (3)	18 200±2 200 (3)	262±31 (3)
Northern toad	*Bufo americanus*	779±56 (12)	10·9±0·66 (12)	−	−
Western alligator lizard	*Gerrhonotus multicarinatus multicarinatus* Blainville	BLD	−	BLD	−
Timber rattlesnake	*Crotalis horridus horridus*	BLD	−	−	−
Garter snake	*Thamnophis sirtalis sirtalis* L.	−	−	−	−
Rabbit	*Oryctolagus* sp.	213	3·02	5 970	84·9
Opossum	*Didelphis virginiana*	437	5·4	8 610	105
Kinkajou	*Potos caudivolvulus*§	384	3·56	−	−
Rat	*Rattus norvegicus*	599±49 (5)	8·04±1·08 (5)	22 800±720 (5)	331±19 (4)
Big brown bat	*Eptesicus fuscus*	2 130	17·4	25 200	206
Little brown bat	*Myotis lucifugus*	2 800	26·2	53 400	498
Goat	*Capra hircus*	487	7·46	23 700	362
Rhesus monkey	*Macaca rhesus*	422	5·74	14 000	190
Man	*Homo sapiens*‖	240±17 (2)	3·13±0·05 (2)	8 460±550 (2)	111±1 (2)

Rhesus monkey	*Macaca rhesus*	422	5·74	14 000		190	
Man	*Homo sapiens*‖	240±17 (2)	3·13±0·05 (2)	8 460±550 (2)		111±1 (2)	

Table 1 (*cont.*)

		Arginine-synthetase system		Argininosuccinate cleavage enzyme		Arginase	
Common name	Binominal name	Units/g.	Units/mg. of protein	Units/g.	Units/mg. of protein	Units/g.	Units/mg. of protein
Mudpuppy	*Necturus maculosus maculosus* Rafinesque	9·1±1·0 (3)	0·26±0·05 (3)	56·1	1·37	20 000±13 100 (3)	502±360 (3)
Salamander	*Ambystoma tigrinum*	–	–	–	–	–	–
Newt	Not identified	–	–	>121	–	–	–
Congo eel	*Amphiuma means*	29·8	0·73	369	9·03	18 700	371
Tadpole	*Rana catesbeiana**						
	Stage VIII	–	–	19	0·75	–	–
	Stages III–X	<2·6	<0·04	9·61	0·23	–	–
	Paddle stages	–	–	25·1	0·42	–	–
	Stage XVIII	1·9	0·05	–	–	–	–
	Stage XIX	5·0	0·13	–	–	–	–
	Stages XI–XIX	–	–	>52	–	–	–
	Stage XXV	18·6	0·66	–	–	–	–
Bullfrog	*Rana catesbeiana*	46·3±9·9 (4)	0·69±0·22 (3)	498†	5·6	24 600	272
Leopard frog	*Rana pipiens*	33·8	0·65	–	–	–	–
Painted turtle	*Chrysemys picta*	44·9	–	–	–	6 030	–
Fowler's toad	*Bufo fowleri*	–	–	–	–	–	–
Giant toad	*Bufo marinus*	33·2±6·0 (3)	0·60±0·11 (3)	–	–	22 500±3 560 (3)	330±79 (3)
Northern toad	*Bufo americanus*	33·0±13·8 (12)	0·50±0·19 (12)	–	–	–	–
Western alligator lizard	*Gerrhonotus multicarinatus multicarinatus* Blainville	BLD	–	–	–	5–30‡	–
Timber rattlesnake	*Crotalis horridus horridus*	BLD	–	BLD	–	–	–
Garter snake	*Thamnophis sirtalis sirtalis* L.	–	–	–	–	78±22 (2)‡	0·79±0·19 (2)
Rabbit	*Oryctolagus* sp.	25·6	0·45	175	3·1	13 300	189
Opossum	*Didelphis virginiana*	–	–	–	–	–	–
Kinkajou	*Potos caudivolvulus*§	7·9	0·08	–	–	–	–
Rat	*Rattus norvegicus*	85·2±4·2 (5)	1·32±0·08 (5)	150±64 (2)	1·6±0·5 (2)	29 800±10 800 (5)	420±32 (5)
Big brown bat	*Eptesicus fuscus*	>47·2	>0·42	–	–	26 300	215
Little brown bat	*Myotis lucifugus*	>90·6	>0·91	–	–	52 200	487
Goat	*Capra hircus*	>71·3	>1·16	–	–	8 800	135
Rhesus monkey	*Macaca rhesus*	40·9	0·65	–	–	11 200	152
Man	*Homo sapiens*‖	51·6±3·4 (2)	0·775±0·005 (2)	174±6 (2)	2·64±0·26 (2)	7 750	108

* Larval development is divided into twenty-five morphological stages.
† Assay was performed on combined cetyltrimethylammonium bromide extracts (modification).
‡ Water homogenate; all other determinations were made on cetyltrimethylammonium bromide homogenates.
§ Frozen liver.
‖ Adult females; analyses were performed in collaboration with Dr A. L. Kennan.

The results of a typical experiment for one of the assays are given for illustrative purposes. When *Necturus* liver was assayed for ornithine transcarbamylase, 2·10 μmoles of citrulline were produced in 30 min. at 38° with extract containing 84 μg. of protein; the amounts of citrulline (in μmole) produced under the following conditions were: boiled enzyme, 0·35; no extract, 0·28; no ornithine, 0·22; no carbamyl phosphate, 0·04 (slight residual activity is attributed to non-enzymic transcarbamylation). The production of citrulline was linear with time over a 40 min. period.

Urea-cycle enzymes have been observed in all species of the Anura, Urodela and Mammalia examined to date. In addition, the painted turtle (terrapin), *Chrysemys picta*, a representative species of the chelonian reptiles, also possessed these enzymes. Carbamyl phosphate synthetase, the arginine-synthetase system and the argininosuccinate-cleavage enzyme could not be demonstrated in the liver of the timber rattle-snake, *Crotalus horridus horridus*, and studies with the livers of pigeon and sparrow indicated that the enzymes of the cycle in these were also below the limit of detection by the assay procedures (cf. Grisolia & Cohen, 1953). These findings are consistent with previous studies on the distribution of ammono-, ureo- and urico-telism among various groups of animals (Needham, 1931; Prosser, 1950). Thus birds and reptiles, except certain turtles (Moyle, 1949), are uricotelic, amphibians and mammals are ureotelic and the bony fish, the Teleostei, are ammonotelic. It is known that carbamyl phosphate-synthetase and ornithine-transcarbamylase activities are below the present limits of detection in the livers of the last-named. W. D. Brown and G. H. Burnett, in the authors' Laboratory (unpublished work), could demonstrate the presence of neither carbamyl phosphate synthetase nor ornithine transcarbamylase in acetone-dried powders of the livers of Holostei, e.g. the gar pike (*Lepidosteus osseus oxyurus*) and the bowfin (*Amia calva*), nor in the Teleostei, e.g. the perch (*Perca flavescens*), the carp (*Cyprinus carpio*) and the brown trout (*Salmo trutta fario*).

All of the urea-cycle enzymes could be demonstrated in the livers of rabbits, monkeys, humans, bats, the South American kinkajou and the opossum. The activities of the enzymes of the bats, *Eptesicus fuscus* and *Myotis lucifugus*, were especially high. The liver of the former had a value for carbamyl phosphate synthetase of 2130 units/g. Activity could be demonstrated in bat liver that had been immediately frozen and stored at $-18°$ for 1 week.

Elasmobranchs contain a high concentration of urea in the blood which serves in an osmotic sense to maintain near tonicity with sea water (see Baldwin, 1949; Prosser, 1950). Baldwin (1958) has found that all enzymes of the cycle, with the exception of carbamyl phosphate synthetase, are present in acetone-dried powders of the livers of a variety of Pacific cartilaginous fishes. Some studies performed in 1956 (Brown, 1959; cf. Cohen & Brown, 1959) also provided evidence for the occurrence of ornithine transcarbamylase in acetone-dried powders of shark liver, but no activity could be demonstrated for carbamyl phosphate synthetase.

Carbamyl phosphate-synthetase activity could not be demonstrated in several samples of liver from the shark, *Squalis sucklii* Bernard. In the assays, some spurious colour developed during the determination for citrulline; the nature of this chromogenic substance is unknown at present. If carbamyl phosphate is indeed formed by shark liver, the enzyme could not be detected by the non-isotopic procedure used here for demonstrating this enzyme activity in the liver of other ureotelic vertebrates. A final answer must await assays by the more sensitive isotopic methods. While this manuscript was in preparation, a brief abstract by Anderson & Jones (1959) appeared in which they claimed to have demonstrated carbamyl phosphate-synthetase activity in *Squalis* sp. Our failure to detect the enzyme in *S. sucklii* was surprising, for carbamyl phosphate-synthetase activity has always been observed in the liver of adult animals whenever activity for ornithine transcarbamylase could be demonstrated (see below).

Ornithine transcarbamylase was easily demonstrable in liver preparations from *S. sucklii*. The production of citrulline by a water extract of a potassium chloride-prepared pellet is shown in Fig. 3. This observation confirms the findings of Brown (1959) and of Baldwin (1958) on the occurrence of this enzyme in elasmobranch liver and demonstrates further the substrate requirements, the heat inactivation of the enzyme and the occurrence of the enzyme in the 4000 ***g*** fraction. The activity of the enzyme as a function of pH in 0·045 M-glycylglycine buffer is shown in Fig. 4. The optimum pH of 8·2 and the general shape of the curve corresponds to that observed by Burnett & Cohen (1957) with partially purified ox-liver ornithine transcarbamylase.

Carbamyl phosphate-synthetase activity in relation to amphibian development. *Necturus* is a neotenic, perennibranchiate salamander that has carried over certain larval characteristics (e.g. external gills) to the adult stage. Of the various ureotelic animal livers assayed, that of *Necturus* possessed the lowest levels of carbamyl phosphate synthetase as well as of ornithine transcarbamylase and of the

arginine-synthetase system. The highest level of carbamyl phosphate synthetase observed with *Necturus* was 22 units/g., which is below the average value of 360 ± 80 units/g. observed with seven salamanders of the genus *Ambystoma*. The low value observed with *Necturus* liver is reminiscent of the values for carbamyl phosphate synthetase found with incompletely metamorphosed frog tadpoles of *Rana catesbeiana* (Brown & Cohen, 1958, 1959*b*). Thus the level of this enzyme again shows a correlation with the degree of metamorphosis which an amphibian undergoes.

Amphiuma means is an eel-shaped, largely aquatic amphibian which possesses miniature paired limbs. The adult *Amphiuma* is a form which is at a later stage of urodele ontogeny than is *Necturus* (Noble, 1931). Two specimens of *Amphiuma* showed activity of 175 and 238 units/g. for carbamyl phosphate synthetase, somewhat higher than for *Necturus* and about equal to that of *Ambystoma*, but considerably less than that for the Anura. Frogs and toads, which have attained a greater degree of development than other amphibians, exhibited activities from about 700 to 1000 units/g. The last-named values are of the order of those observed in rat liver.

The level of carbamyl phosphate synthetase can be correlated with the extent of amphibian development. Thus the level of this enzyme increases with the extent of development in this order: *Necturus, Amphiuma, Ambystoma, Rana* $\simeq$ *Bufo*.

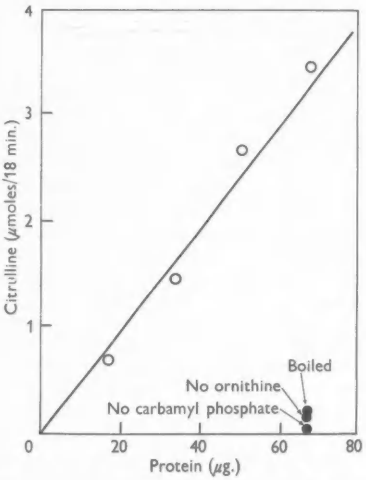

Fig. 3. Ornithine transcarbamylase from liver of *Squalis sucklii* Bernard. Rate of production of citrulline and protein concentration.

Arginase activity of the liver of saurian reptiles. Extracts prepared from the liver of the western alligator lizard, *Gerrhonotus multicarinatus multicarinatus* Blainville, showed a low but significant amount of arginase activity. The results of a typical experiment with this animal are given in Table 2.

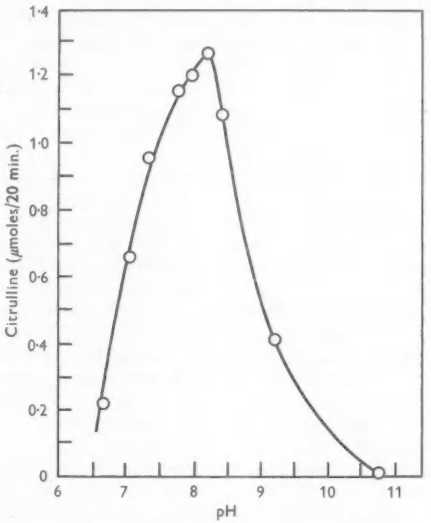

Fig. 4. Optimum pH for ornithine transcarbamylase of *Squalis acanthius*. The system contained: 33 μg. of protein: water extract of KCl-prepared pellet; 1 ml. incubation volume (modification); glycylglycine buffer, 0·045 M.

Table 2. *Arginase activity of the liver of the lizard* Gerrhonotus multicarinatus multicarinatus *Blainville*

A 10% water homogenate (0·4 ml. containing 8·8 mg. of protein/ml.) was incubated for 1 hr. at 38° in the presence of: arginine, pH 9·5, 25 μmoles; glycine buffer, pH 9·5, 50 μmoles; $MnCl_2$, 0·5 μmole; water to give a final volume of 2 ml. (complete system). When solutions of components of the reaction mixture were omitted, an equivalent volume of water was added to bring the final volume to 2 ml. Tubes with urease contained 1 mg. of partially purified urease. Reactions were stopped with 5 ml. of 0·5 M-perchloric acid and the clear supernatant solution after centrifuging was analysed for urea with 1-phenyl-1:2-propanedione-2-oxime. Figures given are for duplicate assays.

System	Urea produced (μmoles/hr.)
Complete	2·17, 2·02
Mn^{2+} ions omitted	1·12, 1·13
Arginine omitted	0·02, 0·01
Complete but with boiled extract	0·04, 0·04
Urease added	0, 0

A value of $18{\cdot}9 \pm 0{\cdot}7$ units/g. was observed for the liver of this specimen. Omission of the substrate, arginine, from the assay system, boiling of the extract or carrying out the incubation in the presence of urease (to destroy any urea produced) led to a negligible amount of product. A partial dependence upon manganese was also demonstrated. In a similar type of experiment, arginase activity was demonstrated for garter-snake liver, two specimens yielding 56 and 99 units/g. Several species of lizards have now been shown also to possess liver-arginase activity (G. W. Brown & P. P. Cohen, unpublished work). Heretofore such activity was thought to be absent from these vertebrates (Clementi, 1915).

N-Acetyl-L-glutamate as cofactor for carbamyl phosphate synthetase. Vertebrate carbamyl phosphate synthetase requires as a cofactor a low-molecular-weight acyl derivative of L-glutamate (Grisolia & Cohen, 1953). The following (in μmoles/ml.) would not replace *N*-acetyl-L-glutamate with frog carbamyl phosphate synthetase: acetylglycine, 20; folic acid, 5; *p*-aminobenzoyl-L-glutamate, 5; glycylglycine, 5; acetamide, 2. The following did not compete with *N*-acetyl-L-glutamate at comparable levels: acetylglycine; *p*-aminobenzoyl-L-glutamate; acetamide. The persistence of the requirement for *N*-acetyl-L-glutamate among the ureotelic vertebrates suggests that carbamyl phosphate synthetase underwent little, if any, structural modification at the active site on the enzyme protein throughout the course of vertebrate evolution.

Nitrogen excretion. In several cases the nitrogen partition of the bladder fluid of certain animals was determined. One specimen of *Bufo fowlerei* showed 24 μmoles of urea/ml. of fluid, corresponding to 98% of the ammonia nitrogen plus urea nitrogen. Bladder fluid of two specimens of *Bufo marinus* contained 92·5 and 89·4% of the total excretory nitrogen (ammonia + urea) as urea nitrogen. The percentage of urea nitrogen in several specimens of *Ambystoma tigrinum* was between 87 and 97. One bladder-fluid sample from an *Amphiuma* contained 3·2 and 14·4 μmoles of ammonia and urea respectively/ml. of fluid, corresponding to 90% of excretory nitrogen as urea nitrogen. To our knowledge this is the first report on this nitrogen partition in the urine of this species. The observed nitrogen partitions confirm the view that these amphibians are ureotelic in the adult form.

Evolutionary significance of the findings

The acquisition of new enzymes by mutation is considered to play a significant role in the evolution of new species. But the deletion of enzymes (Potter, 1957) must also be considered as contributing to the route for the origin of new species. Both types of enzymic alterations, when otherwise not lethal, tend to increase the likelihood that such altered forms will extend their ecological niche (or assume a new one). The thesis is advanced that both types of enzymic alterations, i.e. acquisition and deletion, when related to a given function work in concert in serving the overall economy of the organism. For example, we can consider that the deletion of the urea-cycle enzymes but with acquisition, retention and/or augmentation of those enzymes involved in uric acid synthesis permitted a greater freedom from an aqueous, external environment in embryonic life (Needham, 1942). The possession of a system for the synthesis of uric acid may be looked upon as an enzymic preadaptation, which can be brought into play if and when loss of urea synthesis subsequently occurs. This presumably occurred during evolution of the reptiles. On the other hand, loss of a functioning urea-synthesizing system in certain primitive fishes without concomitant enhancement of net uric acid synthesis appears to be responsible for the excretion by the Teleostei of much of their waste nitrogen as ammonia. Teleosts have made inroads, however, on another form of nitrogen excretion, notably that of trimethylamine oxide (Grollman, 1929).

Although it is not possible at present to consider all of the possibilities of deletion and acquisition of urea-cycle enzymes throughout the sequence of

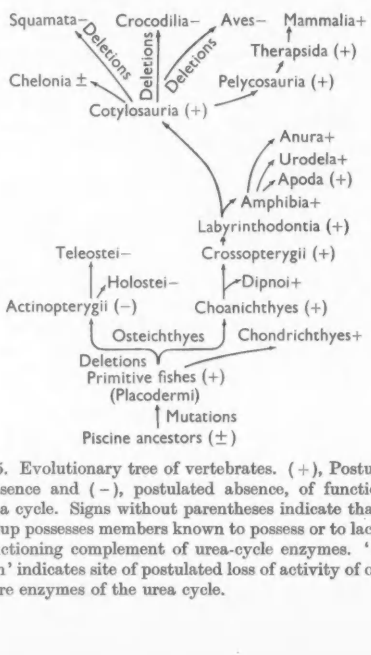

Fig. 5. Evolutionary tree of vertebrates. (+), Postulated presence and (−), postulated absence, of functioning urea cycle. Signs without parentheses indicate that the group possesses members known to possess or to lack the functioning complement of urea-cycle enzymes. 'Deletion' indicates site of postulated loss of activity of one or more enzymes of the urea cycle.

evolution of the vertebrates, certain assumptions can be made which offer a tentative explanation for the distribution of urea-cycle enzymes in the livers of extant vertebrates. It seems advisable to restate the sequence of evolution of nitrogen-excretion mechanisms in the light of the present findings and those presented elsewhere (Needham, 1931; Baldwin, 1949).

The evolutionary tree, as modified from several sources (Noble, 1931; Romer, 1945, 1956), from piscine ancestors to the higher vertebrates, is shown in Fig. 5. The picture which emerges is this: at some time there arose primitive fishes, possibly the Placodermi, possessing probably the complement of urea-cycle enzymes; these primitive fishes were derived from ancestors which possessed enzymes of the urea cycle. As depicted elsewhere (Brown & Cohen, 1958; Ratner, 1955), the urea cycle can operate as such in the intact animal presumably only with the co-operation of four ancillary reactions providing a source of aspartate for the synthesis of argininosuccinate. Thus at least nine enzymes, and presumably at least nine genetic loci, were required before the urea cycle could function as a mechanism for the disposal of ammonia as urea; it seems illogical to assume that these enzymes arose in one evolutionary step, but rather that the evolution of the cycle occurred in stages.

That enzymes of the urea cycle were being exploited in Nature before the evolution of the cycle as a mechanism for the excretion of nitrogen is shown by the widespread occurrence of such enzymes in a variety of plant, animal and bacterial sources (see Brown & Cohen, 1958; Ratner, 1954, 1955). It appears likely that the final acquisition of the complement of urea-cycle enzymes was a process involving a series of mutations serving the economy of primitive and diverse forms in various ways. Thus the enzymes preceding arginase in the cycle offer a route for the synthesis of arginine for protein synthesis and for synthesis of creatine and arginine phosphate.

The assignment of an active urea cycle with its complement of enzymes to certain primitive fishes is based upon the ureotelism exhibited by sharks (of Chondrichthyes), which derived at about the same time from the same general stock as did the Actinopterygii (ray-finned fishes) and the Choanichthyes (possessing internal nostrils associated with lung breathing). It is suggested that certain deletions of enzymes of the urea cycle occurred in the evolvement of the Actinopterygii, for the modern Holostei and Teleostei do not possess all of the enzymes of the urea cycle and are ammonotelic. The term deletion is to be interpreted to mean the lowering of enzymic activity to such a level that the urea cycle no longer functions to any significant extent as a system for the disposal of ammonia as urea. In the instances cited, it is not known whether or not the absence of activity can be attributed to the loss of primary genetic information for the synthesis of individual enzymes. Choanichthyes, the other major subclass of Osteichthyes, is assigned all of the urea-cycle enzymes, as are the later Crossopterygii (Middle Devonian bony fishes) and Labyrinthodontia (primitive amphibians), since higher branches of this stem, e.g. the dipnoans (lungfishes) and amphibians, are ureotelic.

The Cotylosauria ('stem reptiles') are also assigned an active urea cycle since an early branch, the Chelonia, possesses members containing the urea-cycle enzymes, e.g. *Chrysemys* (cf. Moyle, 1949, for ureotelic species). The reptilian Pelycosauria and Therapsida, antecedent to Mammalia, are derived from the main stem stock of reptiles and would be thought still to possess the urea-cycle enzymes. Deletions within the stem reptiles are presumed to have occurred, for Aves, Crocodilia and Squamata do not excrete appreciable amounts of urea, nor are they known to possess the complement of urea-cycle enzymes. The assignment of urea-cycle activity in extinct forms can, of course, come only from indirect evidence. Knowledge on the occurrence or absence of urea-cycle enzymes in the livers of species occupying peculiar phylogenetic positions such as the platypus, coelacanths and the New Zealand sphenodon, could provide valuable information.

Emphasis on net uric acid synthesis is presumed to have occurred sometime during early reptilian development, for chelonian reptiles have the ability to excrete their nitrogen as ammonia, urea and/or uric acid, depending upon species (Moyle, 1949; Khalil & Haggag, 1955) and possibly on environment (Clementi, 1952).

Arginase shows marked persistence throughout the course of vertebrate evolution, for birds (uricotelic) contain arginase in the kidney (Clementi, 1915), although at a lower activity than is found in the livers of amphibians or mammals. This enzyme is also found in the livers of the Teleostei to varying degrees (Hunter & Dauphinee, 1924). For 45 years arginase has been thought to be absent from squamatine liver (Clementi, 1915). Our observations on the occurrence of arginase in the liver of the western alligator lizard and the garter snake (see above) as well as in several other species of lizards (unpublished work) are, we believe, the first observed exceptions to the rule of Clementi that arginase is absent from the liver of such uricotelic vertebrates. The activities of liver arginase in descending order in the vertebrates appear to be sharks $\cong$ frogs, turtles, lizards $\cong$ snakes, birds (absent entirely?). That the level of liver arginase tends to decrease in this order supports the view

that there has been a progressive loss in liver-arginase activity during the course of sauropsidian evolution.

Alternative hypotheses can certainly be offered to explain the enzymic alterations of the urea-cycle enzymes in the evolutionary tree. For example, there could have been deletions and back-mutations (to regain the enzymes) of certain enzymes of the urea cycle almost anywhere in the scheme. Whereas such changes indeed may have occurred, the suggestion at present of such changes seems unwarranted; only a simple and tentative hypothesis consistent with our present state of knowledge has been made.

Smith (1953) has already suggested that urea was the principal nitrogenous end product of protein degradation in Therapsida and Crossopterygii. Smith's conclusions were based, among other things, on renal structure, function and osmotic balances as derived from the contributions of palaeontology and comparative anatomy. Our independent conclusion is based upon the presence of urea-cycle-enzyme activities in elasmobranchs, amphibians, certain chelonian reptiles and mammals, and the phylogenetic relationship to these of the therapsids and the crossopterygians. Contrary to Smith, however, we do not classify teleosts as ureotelic because (i) only small quantities of urea are excreted by teleosts, compared with ammonia, creatinine, trimethylamine and other unidentified nitrogenous products, depending upon species (cf. Prosser, 1950), and (ii) all experimental evidence for the occurrence of the ornithine–urea cycle in teleosts is negative.

Comments pertaining to acquisition and deletion of enzymes in relation to the occurrence or absence of enzymes in extant vertebrates is not to be confused with the phenomenon of the induction of the urea cycle in tadpoles which takes place during metamorphosis (Brown & Cohen, 1958, 1959*b*). The genetic pattern for synthesis of urea-cycle enzymes is present in premetamorphic tadpoles but is not manifested in its entirety until the onset of metamorphosis. It may be thought that the genetic pattern for all urea-cycle enzymes is present in the bird egg but that deletion during ontogeny results in the loss of all of the urea-cycle enzymes (except arginase) sometime before hatching. Such an idea of recapitulation was popular for a while and was based on a graph of nitrogen-excretion products in the developing chick embryo published by Needham (1931). This graph has appeared in at least five other books (Baldwin, 1949, 1957; Florkin, 1947; Moody, 1953; Prosser, 1950). But Needham, Brachet & Brown (1935) long ago concluded, and more recently it has been shown (Eakin & Fisher, 1958; Ricceri, 1957), that during embryonic development the bird does not go through an evolutionary recapitulation from ammonia to urea to uric acid excretion, with the resulting temporary acquisition of urea-cycle enzymes. The formation of urea in chick embryos finds explanation in the action of arginase on arginine derived from yolk proteins. Gordon (1956) revived this idea of recapitulation and offered evidence for the occurrence of carbamyl phosphate synthetase and ornithine transcarbamylase in 9-day-old chick embryos. An attempt to repeat this experiment in the authors' Laboratory was unsuccessful.

In view of the fact that a low but significant level of arginase activity has now been observed in lizard and snake liver, where previously it was thought to be absent, and because of conflicting reports of arginase activity in adult avian liver (Chaudhuri, 1927; Clementi, 1946), the status of the occurrence of liver arginase needs to be evaluated again. It is unlikely that such a study would change the general conclusions about the phylogenetic distribution of nitrogen excretion patterns. Studies of this kind, however, might provide information about the nature and extent, and possibly the evolutionary loci, of enzymic alterations which have yielded the excretion patterns in extant species.

SUMMARY

1. Activities of urea-cycle enzymes in the livers of a variety of lower and higher vertebrates are presented. All amphibians and mammals studied possessed the urea-cycle enzymes, as did the painted turtle (terrapin), *Chrysemys picta*.

2. The activity of carbamyl phosphate synthetase reflects the extent of amphibian development, showing an increase in activity with greater development.

3. A scheme is presented of the phylogenetic relationships of extinct and extant vertebrate groups with indication of stages in the evolutionary sequence where deletions of urea-cycle enzymes could have occurred.

4. Data are presented on the occurrence of ornithine transcarbamylase in shark liver.

5. Of all the animals investigated, bats possessed the highest levels of carbamyl phosphate synthetase.

6. An exception to the rule of Clementi, namely that arginase is absent from the livers of uricotelic animals, has been found. Arginase activity was demonstrated in lizard and snake liver.

We are indebted to Dr William G. Reeder of the Department of Zoology for his valuable comments and suggestions on our phylogenetic chart and to Dr W. Welker of the Department of Physiology, who supplied us with kinkajou, opossum, bat and rattlesnake livers. We wish to thank Drs A. W. Martin and D. D. Chapman of the Department of

Zoology, University of Washington, Seattle, for extending to one of us (G. W. B.) facilities for carrying out studies with shark liver at the Oceanographic Laboratory in Seattle and at the Marine Biological Station at Friday Harbor in August 1958. Appreciation is also expressed to Dr John C. Neess of the Department of Zoology for classifying several of the specimens for us.

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REFERENCES

Anderson, A. D. & Jones, M. E. (1959). *Abstr.* 135th *Meet. Amer. chem. Soc.: Div. biol. Chem.* no. 126.

Baldwin, E. (1949). *An Introduction to Comparative Biochemistry*, 3rd ed. Cambridge University Press.

Baldwin, E. (1957). *Dynamic Aspects of Biochemistry.* Cambridge University Press.

Baldwin, E. (1958). *Nature, Lond.*, **181**, 1591.

Brown, G. W., jun. & Cohen, P. P. (1958). In *A Symposium on the Chemical Basis of Development*, p. 495. Ed. by McElroy, W. D. & Glass, B. Baltimore: The Johns Hopkins Press.

Brown, G. W., jun. & Cohen, P. P. (1959*a*). *J. biol. Chem.* **234**, 1769.

Brown, G. W., jun. & Cohen, P. P. (1959*b*). *J. biol. Chem.* **234**, 1775.

Brown, W. D. (1959). M.D. Thesis: University of Wisconsin Medical School.

Burnett, G. H. & Cohen, P. P. (1957). *J. biol. Chem.* **229**, 337.

Chaudhuri, A. C. (1927). *Brit. J. exp. Biol.* **5**, 97.

Clementi, A. (1915). *Arch. Fisiol.* **13**, 189.

Clementi, A. (1946). *Boll. Soc. ital. Biol. sper.* **22**, 1075.

Clementi, A. (1952). *Arch. Sci. biol., Napoli*, **36**, 10.

Cohen, P. P. & Brown, G. W., jun. (1959). In *Comparative Biochemistry*, Ed. by Florkin M. & Mason, H. S. New York: Academic Press Inc. (in the Press).

Eakin, R. E. & Fisher, J. E. (1958). In *A Symposium on the Chemical Basis of Development*, p. 514. Ed. by McElroy, W. D. & Glass, B. Baltimore: The Johns Hopkins Press.

Florkin, M. (1947). *L'Évolution Biochimique*, 2nd ed. Paris and Liège: Masson et Cie and Desoer.

Gordon, M. W. (1956). In *Progress in Neurobiology. Vol.* 1: *Neurochemistry.* Ed. by Korey, S. R. New York: P. B. Hoeber.

Grisolia, S. & Cohen, P. P. (1953). *J. biol. Chem.* **204**, 753.

Grollman, A. (1929). *J. biol. Chem.* **81**, 267.

Hunter, A. & Dauphinee, J. A. (1924). *Proc. Roy. Soc.* B, **97**, 227.

Khalil, F. & Haggag, G. (1955). *J. exp. Zool.* **130**, 423.

Kim, S. (1959). Ph.D. Thesis: University of Wisconsin Medical School.

Krebs, H. A. & Henseleit, K. (1932). *Hoppe-Seyl. Z.* **210**, 33.

Moody, P. A. (1953). *Introduction to Evolution.* New York: Harper and Bros.

Moyle, V. (1949). *Biochem. J.* **44**, 581.

Needham, J. (1931). *Chemical Embryology*, vol. 2. Cambridge University Press.

Needham, J. (1942). *Biochemistry and Morphogenesis.* Cambridge University Press.

Needham, J., Brachet, J. & Brown, R. K. (1935). *J. exp. Biol.* **12**, 321.

Noble, G. K. (1931). *The Biology of the Amphibia.* New York: McGraw-Hill Book Co. Inc.

Potter, V. R. (1957). *Med. Bull. Univ. Mich.* **23**, 401.

Prosser, C. L. (1950). In *Comparative Animal Physiology*, p. 187. Ed. by Prosser, C. L. Philadelphia: W. B. Saunders Co.

Ratner, S. (1954). *Advanc. Enzymol.* **15**, 319.

Ratner, S. (1955). In *A Symposium on Amino Acid Metabolism*, p. 231. Ed. by McElroy, W. D. & Glass, H. B. Baltimore: The Johns Hopkins Press.

Ricceri, G. (1957). *Ital. J. Biochem.* **6**, 353.

Romer, A. S. (1945). *Vertebrate Paleontology.* Chicago: University Press.

Romer, A. S. (1956). *A Shorter Version of the Second Edition of the Vertebrate Body.* Philadelphia: W. B. Saunders Co.

Smith, H. W. (1953). *From Fish to Philosopher.* Boston: Little, Brown and Co.

Biochem. J. (1960) **75**, 91

Studies on the Chemical Basis of the Antigenicity of Proteins

1. ANTIGENICITY OF POLYPEPTIDYL GELATINS

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Most proteins have been tested for antigenicity, and practically all have been found to be antigenic, though to a varying extent. Although antigens of non-protein nature are also known, the proteins seem to be the most abundant and important antigenic substances yet studied. It is the purpose of this series of investigations to try to establish some correlation between the chemical nature of the protein antigens and their immunological activity.

To be antigenic, a molecule must possess a certain minimal degree of complexity and a certain mole-

cular size. On the other hand, it seems that only a part of the protein molecule is concerned with its antigenic specificity. The questions arise: what chemical structure must be present in the protein molecule to endow it with antigenic properties and what is the chemical nature of the moieties that can either enhance its antigenicity or influence its antigenic specificity?

These problems may be approached either by the immunological study of the products of partial degradation of antigen molecules or, as in the work reported here, by the investigation of the changes in the serological activity and specificity resulting from the attachment to the protein molecule of groups which may play a role in antigenicity.

Our detailed knowledge of haptenic specificity is due mainly to the pioneering work of Landsteiner (1945). In most studies the haptenic groups were derived from compounds not present normally in a protein, or, where they were composed of natural amino acids, the haptene was bound to the antigen through an azo bond (Landsteiner & van der Scheer, 1939). Serological investigations of substances prepared by attachment to proteins, through peptide bonds, of polypeptide chains composed of naturally occurring amino acids, would be of interest.

Polypeptidyl proteins may be obtained by causing proteins to react under mild conditions (aqueous solutions, neutral pH, low temperatures) with *N*-carboxy-α-amino acid anhydrides (Becker & Stahmann, 1953; Sela, 1954). In this way peptides of different sizes can be built on the free amino groups of the protein (Katchalski & Sela, 1958).

Immunological studies of polypeptidyl derivatives of strongly antigenic proteins, prepared by this method, have been reported recently. Thus the introduction of 171 and 210 glycyl units into bovine serum albumin produced a small but definite change in specificity (Makinodan, Becker, Wolfe & Stahmann, 1954). Stahmann, Tsuyuki, Weinke, Lapresle & Grabar (1955) investigated, by means of double diffusion in gels and by immunoelectrophoresis, the antigenic properties of polyleucyl, polyphenylalanyl, polyglutamyl and polylysyl derivatives of rabbit and bovine serum albumin. The immune sera prepared with polypeptidyl rabbit serum albumins cross-reacted with bovine serum albumin.

It has been considered whether the addition of polypeptides of different chemical composition to an immunologically rather inert protein carrier could enhance its low antigenicity and in this paper the first results of an immunological investigation of polypeptidyl derivatives of gelatin are reported.

The antigenicity of poly-α-amino acids has been investigated by several methods. Stahmann *et al.* (1955) reported that polyglutamic acid and polylysine are antigenic for the rabbit, though the antibodies could not be precipitated by the homologous antigens. On the other hand, Maurer (1957) did not observe detectable antibodies against polyglutamic acid in man, rabbit or guinea pig. Similarly, attempts to produce antibodies against various synthetic polymers of a single amino acid, by methods both *in vitro* and *in vivo*, were unsuccessful (Maurer, Subrahmanyam, Katchalski & Blout, 1959). Sela & Haurowitz (1958) failed to detect in rabbits antibodies to poly-*p*-arsanilazo-L-tyrosine by the usual precipitation techniques. Thus it seems that most poly-α-amino acids are not antigenic (Sela & Katchalski, 1959).

Gelatin is such a poor antigen that for many years its antigenicity escaped notice (Haurowitz, 1950; Boyd, 1956) and could be demonstrated only with refined precipitin techniques (Maurer, 1954*a*, *b*, 1955). A number of possible reasons for this low antigenicity may be postulated.

As a rigid structure seems to be essential for the antigenicity of proteins, it is quite possible that the lack of antigenicity of gelatin could be due to the lack of a definite structure in this heat-denatured protein, containing large amounts of glycine. The high percentage of proline and hydroxyproline could also be responsible for the lack of a configurational structure of the type encountered in other proteins (Szent-Gyorgyi & Cohen, 1957; Harrington & Sela, 1958). The deficiency of aromatic groups in gelatin has led many authors to postulate the necessity of such groups for antigenicity. As a matter of fact, the attachment of *O*-β-glucosido-*N*-benzyloxycarbonyl tyrosine (Clutton, Harington & Yuill, 1938) or *N*-benzyloxycarbonyltyrosine (Humphrey & Yuill, 1939; Micheel & Schallenberg, 1952) to gelatin gave substances which elicited the production of antibodies. These could not be precipitated with homologous antigens, but could be detected by precipitation with the respective derivatives of globulin. The benzyloxycarbonyl groups were not removed from the above antigens before the serological tests.

The possible enhancement of the antigenicity of gelatin by the attachment of tyrosine peptides was investigated by Sela, Katchalski & Olitzki (1956). Typical anaphylactic shocks were shown in guinea pigs sensitized with polytyrosyl gelatin (Sela, 1954), after intracardial injection of the same compound. On the other hand, no sensitization was observed by a similar treatment with gelatin alone or with a copolymer of tyrosine and aspartic acid. Poly-*p*-arsanilazotyrosyl gelatin acts as a strong antigen in rabbits, as shown by the precipitin test (Sela & Haurowitz, 1958).

The above preliminary investigations prompted us to undertake a systematic survey of the possible enhancement of the antigenicity of gelatin by the attachment of various polypeptidyl chains, which include derivatives of alanine, of the aromatic amino acids tyrosine, tryptophan and phenylalanine and of the strongly polar lysine and glutamic acid as well as of the sulphur-containing cysteine. The aromatic α-amino acids are present in gelatin only to a very small extent and cysteine was studied because gelatin contains almost no sulphur. The charged residues were added to test the common assumption that polar groups are of decisive significance for antigenic specificity. In order to see if the addition of any amino acid chain can enhance the antigenicity of gelatin, the relatively simple amino acid alanine was attached.

The amino acid with which gelatin was enriched was bound, in the form of short polypeptide chains, to as many as 75% of the amino groups of the original protein. Thus the polypeptides are linked to gelatin mainly through peptide bonds involving the ε-amino groups of lysine. Although such peptide bonds are not usually found in proteins, the existence of a peptide chain beginning at an ε-amino group of lysine in collagen has been reported recently (Mechanic & Levy, 1959).

This paper presents evidence that the polytyrosyl, polytryptophyl, polyphenylalanyl and, to some extent, polycysteinyl derivatives of gelatin behave as relatively strong antigens. The addition of polyglutamyl, polylysyl and polyalanyl chains do not significantly increase the antigenicity of gelatin.

EXPERIMENTAL

Materials

Amino acids and peptides are abbreviated as in Sanger (1952): Ala (alanine), Cys (cysteine), Glu (glutamic acid), Lys (lysine), Phe (phenylalanine), Ser (serine), Try (tryptophan) and Tyr (tyrosine). Gelatin (Gel), U.S.P. granular, was obtained from Fischer Scientific Co. Pittsburgh, Pa., U.S.A. After drying over phosphorus pentoxide *in vacuo*, it contained 18·3% of nitrogen (Eastoe, 1955, reported 18·11% for bone gelatin). Egg albumin, twice recrystallized, was prepared according to La Rosa (1927). Egg-white lysozyme, twice recrystallized, was obtained through the kindness of Dr W. F. Harrington. Edestin and the amino acids used in this study were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.

Poly-L-glutamic acid (number-average degree of polymerization 50) was synthesized according to Katchalski & Berger (1957). Multi-chain poly-L-glutamic acid (Yaron & Berger, 1958) of a molecular weight 170 000 was a gift from Mr A. Yaron. Tri-L-tyrosine ethyl ester (Neumann, Levin, Berger & Katchalski, 1959) was a gift from Dr Y. Levin. Glycyl-L-tyrosine was purchased from L. Light and Co. Ltd.

Polypeptidyl gelatins

All the polypeptidyl gelatins (Table 1) were prepared by the method of Becker & Stahmann (1953) as applied to gelatin by Sela (1954). Various *N*-carboxy-α-amino acid anhydrides in dioxan solution (1 g. of anhydride/20 ml. of anhydrous dioxan) were caused to react with gelatin in aqueous solution buffered at pH 7·0 with 0·05 M-phosphate (1 g. of gelatin in 50 ml. of buffer). The reaction was carried out for 24 hr. at 2°, followed by an additional 24 hr. at room temperature.

Poly-DL-alanyl gelatin. This compound (pAlaGel) was prepared by adding 6 m-moles of *N*-carboxy-DL-alanine anhydride (Sela & Berger, 1955)/g. of gelatin. It was purified by dialysis against distilled water at 2° for 3 days. Under the polymerization conditions used, but in the absence of gelatin, *N*-carboxyalanine anhydride gives low-alanine peptides which passed through the dialysing membrane to an extent of 97% (Sela, Katchalski & Gehatia, 1956). The contents of the dialysis bag were lyophilized and stored at 2°. A chromatographic analysis of this material showed no traces of low-molecular-weight peptides. To determine the content of alanine, pAlaGel was hydrolysed with 6 N-hydrochloric acid at 105° for 24 hr. The hydrolysate was subjected to paper electrophoresis on a Whatman no. 1 filter paper in 25 mM-phthalate buffer, pH 5·92 (5·10 g. of potassium hydrogen phthalate and 0·86 g. of sodium hydroxide in 1 l. of water), at a potential gradient of 10 v/cm., at 25°. After 2 hr. the paper was dried and chromatographed in the second dimension in butan-1-ol–acetic acid–water (50:12:50, v/v) for 48 hr. Alanine was estimated by ninhydrin colorimetry (Kay, Harris & Entenman, 1956) (see Table 1). To obtain information on the extent to which the ε-amino groups of gelatin served as initiators in the polymerization of *N*-carboxyalanine anhydride, pAlaGel was dinitrophenylated at pH 9·0 at 40° for 90 min. and hydrolysed. *N*-Dinitrophenyl-DL-alanine and ε-*N*-dinitrophenyl-L-lysine were determined colorimetrically (Levy, 1954; Anfinsen, Sela & Tritch, 1956). The ratio of dinitrophenyl (DNP)-alanine to ε-DNP-lysine is given in Table 1.

Poly-L-cysteinyl gelatin. This compound (pCysGel) was prepared by adding 5 m-moles of *N*-carboxy-L-cysteine anhydride (Berger *et al.* 1958)/g. of gelatin. The reaction mixture was solubilized by the addition of alkali to pH 11·0, followed by neutralization with hydrochloric acid. Poly-L-cysteine is not soluble in water in the neutral pH range (Berger, Noguchi & Katchalski, 1956). pCysGel was purified by dialysis against 0·02 N-sodium thioglycollate for 3 days, followed by dialysis against water for 3 hr. The lyophilized material contained 2·2% of elementary sulphur (corresponding to 7·1% of cysteine residues). The content of sulphydryl groups was estimated spectrophotometrically by a modification (Sela, White & Anfinsen, 1959) of the *p*-chloromercuribenzoate method of Boyer (1954). It corresponded to 6·7% of cysteine residues. pCysGel was kept under vacuum at 2°; its sulphydryl content did not change for several months under these conditions.

Poly-L-glutamyl gelatin. This compound (pGluGel) was prepared by adding 4 m-moles of γ-benzyl-*N*-carboxy-L-glutamate anhydride (Katchalski & Berger, 1957)/g. of gelatin. After the completion of the reaction, 2 vol. of cold acetone was added and the precipitated polybenzylglutamyl gelatin was washed with acetone and dried *in vacuo* over phosphorus pentoxide. The benzyl groups

were removed with anhydrous hydrogen bromide in acetic acid at 2° for 65 hr. (Yaron & Berger, 1958). pGluGel was precipitated with ether, dissolved in water at pH 7·0 (by the cautious addition of N-sodium hydroxide), dialysed, lyophilized and stored as described for pAlaGel. The content of glutamic acid in pGluGel, as well as the ratio of DNP-glutamic acid to ε-DNP-lysine, were determined as for pAlaGel (see Table 1).

The electrophoretic mobility of pGluGel was compared with that of mixtures of gelatin and glutamic acid, and of gelatin and polyglutamic acid. The mixtures contained the same amount of glutamic acid as pGluGel. The measurements were carried out on 1% solutions, in a Kern LK 30 electrophoresis apparatus, in 0·05 M-phthalate buffer, pH 6·0, and 0·05 N-sodium chloride, at a potential gradient of 70 v, at room temperature. pGluGel moved towards the anode as a single peak, and the mixtures of gelatin and glutamic acid or polyglutamic acid separated within 5 min.

Poly-L-lysyl gelatin. This compound (pLysGel) was prepared by adding 5 m-moles of ε-*N*-benzyloxycarbonyl-α-*N*-carboxy-L-lysine anhydride (Bergmann, Zervas & Ross, 1935)/g. of gelatin. After the completion of the reaction 2 vol. of cold acetone was added, and the precipitated polybenzyloxycarbonyl-lysyl gelatin was washed with acetone and dried *in vacuo* over phosphorus pentoxide. The benzyloxycarbonyl groups were removed with anhydrous hydrogen bromide (Ben-Ishai & Berger, 1952) at room temperature for 1 hr. pLysGel was precipitated with ether, dissolved in water at pH 7·0, dialysed, lyophilized and stored as described above. The content of lysine in pLysGel was determined similarly to that of alanine in pAlaGel, except that the chromatography in the second dimension was carried out in butan-1-ol–water–pyridine–acetic acid (30:29:20:6, by vol.).

The electrophoretic mobility of pLysGel was compared with that of a mixture of gelatin and polylysine hydrobromide (Katchalski, 1957) (number-average degree of polymerization 70) containing the same amount of lysine as pLysGel. The measurements were carried out on a Whatman no. 1 filter paper in 0·025 M-phthalate buffer, pH 6·0, at a potential gradient of 10 v/cm., at 25°. After 2 hr. the zones were detected with ninhydrin. pLysGel moved slowly as a round spot towards the cathode, and the mixture separated into a spot of gelatin that stayed at the origin and an elongated spot of polylysine that travelled quickly towards the cathode.

Poly-L-phenylalanyl gelatin. This compound (pPheGel) was prepared by adding 2 m-moles of *N*-carboxy-L-phenylalanine anhydride (Sela & Berger, 1955)/g. of gelatin. Poly-L-phenylalanine is insoluble in water (Katchalski & Sela, 1958). The products of the reaction of gelatin with higher amounts of *N*-carboxy-L-phenylalanine anhydride were similarly insoluble in water. pPheGel prepared as mentioned above could be dissolved by addition of alkali up to pH 11·0. No precipitate was formed upon neutralization. pPheGel was purified by dialysis, lyophilized and stored as usual. To determine the content of phenylalanine, pPheGel was hydrolysed with 6 N-hydrochloric acid at 110° for 36 hr. The hydrolysate was chromatographed in butan-1-ol–acetic acid–water (50:12:50, by vol.) and valine, and phenylalanine plus leucine, were estimated by ninhydrin colorimetry (Kay *et al.* 1956). Since this solvent does not separate the last two amino acids, a similar analysis was performed on unmodified gelatin and the amount of attached phenylalanine was calculated by difference (see Table 1). The ratio of DNP-phenylalanine to ε-DNP-lysine was determined as for pAlaGel.

Poly-L-tryptophyl gelatin. This compound (pTryGel) was prepared by adding 5 m-moles of α-*N*-carboxy-L-tryptophan anhydride (Patchornik, Sela & Katchalski, 1954)/g. of gelatin. pTryGel obtained was solubilized by addition of alkali, followed by neutralization. It was purified by dialysis as usual and lyophilized. The content of tryptophan in pTryGel was calculated from the absorption of the material, as well as of its alkaline (barium hydroxide) hydrolysate, at 280 mμ (Beaven & Holiday, 1952).

Table 1. *Polypeptidyl gelatins*

A	B	C	D	E	F	G	H
Gelatin derivative (enriched with amino acid X)	Percentage of the X-amino acid residue in the original gelatin	Percentage of the X-amino acid residue in the gelatin derivative	Enrichment* $\left(\frac{C-B}{100-C}\right)\times 100$	Moles of amino acid attached/ 100 kg. of gelatin	Ratio of α-amino groups to ε-amino groups†	Number of moles of distinct polypeptide chains/ 100 kg. of gelatin‡ [30·6 F/ (F + 1)]	Average no. of amino acid residues/ peptide chain attached (E/G)
pAlaGel	9·0§; 8·7‖	18·4	11·8	166	3·65	24	6·9
pCysGel	0·0§	7·1	7·6	74	—	—	—
pGluGel	10·2§; 10·1‖	26·6	22·5	175	2·85	23	7·6
pLysGel	3·82§; 4·12‖	22·6	24·1	189	—	—	—
pPheGel	2·11§	8·85	7·4	50	2·45	22	2·3
pTryGel	0·06¶	25·4	34·0	183	—	—	—
pTyrGel	0·21§; 0·42‖	10·3	11·1	68	2·35	21	3·2

* Calculated assuming gelatin as 100%.

† Determined from the absorptions of the DNP-amino acid attached, and of ε-DNP-lysine, recovered from the hydrolysates of the dinitrophenylated gelatin derivatives.

‡ Calculated assuming 30·6 lysine residues/100 kg. of gelatin (Eastoe, 1955).

§ From Eastoe (1955).

‖ Determined by the authors under the same conditions as for the corresponding gelatin derivatives.

¶ From Goodwin & Morton (1946).

Poly-L-tyrosyl gelatin. This compound (pTyrGel) was prepared by adding 2·5 m-moles of *O*-benzyloxycarbonyl-*N*-carboxy-L-tyrosine anhydride (Katchalski & Sela, 1953)/g. of gelatin. After the completion of the reaction 2 vol. of cold acetone was added, and the precipitated poly-*O*-benzyloxycarbonyltyrosyl gelatin was washed with acetone and dried *in vacuo* over phosphorus pentoxide. The benzyloxycarbonyl groups were removed by the action of anhydrous hydrogen bromide in acetic acid (Sela, 1954) at room temperature for 1 hr. pTyrGel was precipitated with ether, dissolved in water at pH 7·0, dialyzed, lyophilized and stored in the cold as usual. Poly-L-tyrosine is not soluble in water in the neutral pH range. The content of tyrosine in pTyrGel was calculated from the absorption of an alkaline solution (pH 13·0) at 293·5 mμ. The ratio of DNP-tyrosine to ε-DNP-lysine was determined as described for pAlaGel (see Table 1).

Poly-DL-seryl gelatin. This compound (pSerGel) was prepared by adding 4 m-moles of *N*-carboxy-DL-serine amino acid anhydride (Berger *et al.* 1958)/g. of gelatin. The reaction product was dialysed and lyophilized. It contained 6·47% of serine residues, as determined by ninhydrin colorimetry after chromatography in pyridine–water (65:35, v/v) of the acid hydrolysate. This corresponds to an enrichment of 3·1%.

Poly-3-(p-arsonylphenylazo)-L-tyrosyl gelatin. This compound (pAzoTyrGel) was prepared similarly to other azo derivatives of polytyrosine (Sela & Katchalski, 1955). Diazotized *p*-arsanilic acid was coupled with a polytyrosyl gelatin containing 16% of tyrosine residues (1 mole of *p*-arsonylphenyl diazonium chloride/mole of tyrosine residue: thus pAzoTyrGel contains 31·4% of *p*-arsonylphenylazotyrosyl residues). pAzoTyrGel was precipitated with dilute hydrochloric acid, dissolved in dilute alkali, purified by dialysis against water and lyophilized. The material contained 5·8% of As (calc. for pAzoTyrGel containing 31·4% of *p*-arsonylphenylazotyrosyl residues of formula $C_{15}H_{13}O_5N_3As$: 6·0% As).

Labelled pTyrGel. This was prepared by iodination of pTyrGel with ^{131}I (50 μc/10 mg.) according to Talmage, Baker & Akeson (1954). The substance, isolated after exhaustive dialysis, had a radioactivity of 800 000 counts/min./mg.

Polypeptidyl egg albumins and edestins

These were prepared and analysed by techniques analogous to those of polypeptidyl gelatins. They are listed in Table 2.

Gelatin treated with anhydrous hydrogen bromide

Gelatin was dried at 60° *in vacuo* over phosphorus pentoxide. The dry sample was dissolved in acetic acid saturated with anhydrous hydrogen bromide. After 65 hr. it was precipitated with ether, dissolved in water at pH 7·0, dialysed as usual and lyophilized.

α-Amino acid copolymers

The *N*-carboxy-α-amino acid anhydride compounds of L-tyrosine (Berger *et al.* 1958), γ-benzyl-L-glutamate (Katchalski & Berger, 1957), L-leucine (Go & Tani, 1939), DL-alanine and L-phenylalanine (Sela & Berger, 1955) were used as starting monomers. They were all recrystallized repeatedly until no chlorine could be detected. In Table 3 are given the various copolymers prepared. The synthesis of tyrosine-containing copolymers was carried out at room

Table 2. *Polypeptidyl derivatives of other proteins*

A Protein derivative (enriched with amino acid X)	B Percentage of the X-amino acid residue in the original protein*	C Percentage of the X-amino acid residue in the protein derivative	D Enrichment† $\left(\frac{C-B}{100-C}\right)\times 100$
Poly-L-phenylalanyl egg albumin	6·82	14·1	8·5
Poly-L-tryptophyl egg albumin	1·10	17·6	20·0
Poly-L-tyrosyl egg albumin	3·32	14·8	13·5
Poly-L-phenylalanyl edestin	4·85	6·75	2·2
Poly-L-tyrosyl edestin	3·83	19·8	19·9

* From Tristram (1953). † Calculated assuming the original protein as 100%.

Table 3. *Copolymers of α-amino acids*

Copolymer of	Molar ratio of *N*-carboxy-α-amino acid anhydride molecules in the polymerization mixture	Molar ratio of the amino acid residues in the copolymer	Number-average degree of polymerization
L-Tyr, L-Glu	1:1	1:1·1	31
L-Tyr, L-Glu	1:3	1:4·0	31
L-Tyr, L-Glu	1:9	1:9·0	88
L-Tyr, DL-Ala	1:9	1:10·1	35
L-Phe, L-Glu	1:1	1:0·87	142
L-Phe, L-Glu	1:3	1:2·1	110
L-Phe, L-Glu	1:9	1:4·2	183
L-Tyr, L-Leu, L-Glu	1:1:4	1:1:1	39
L-Tyr, monoiodotyrosine, di-iodotyrosine, L-Glu	—	17:17:14:52	31

temperature in anhydrous dioxan (1 g. of *N*-carboxy-α-amino acid anhydride/20 ml. of dioxan), triethylamine being used as initiator. The benzylated copolymers were precipitated with water, dried *in vacuo* over phosphorus pentoxide and the benzyl groups were removed with anhydrous hydrogen bromide in acetic acid, as described for pGluGel. The copolymer of Tyr and Ala was precipitated from the polymerization mixture with ether, taken up in water, dialysed against water and lyophilized. The copolymers of phenylalanine and γ-benzylglutamate were prepared in anhydrous benzene and debenzylated by passing a stream of anhydrous hydrogen bromide through the reaction mixtures. The precipitation of the copolymers was completed with anhydrous ether; the copolymers obtained were washed with anhydrous ether and dried *in vacuo* over phosphorus pentoxide and potassium hydroxide.

The amino acid content of the various copolymers was determined by ninhydrin colorimetry (Kay *et al.* 1956) of the hydrolysates after chromatography in butan-1-ol–acetic acid–water (50:12:50, by vol.), or, for tyrosine-containing copolymers, by spectrophotometric analysis at pH 13·0 and 293·5 mμ. The number-average degrees of polymerization were obtained from amino nitrogen (Van Slyke) determinations.

Iodinated copolymer of L-*tyrosine and* L-*glutamic acid* (1:1·1)

This was synthesized similarly to polydi-iodotyrosine (Katchalski & Sela, 1953). The isolated iodinated copolymer was hydrolysed by reflux with saturated barium hydroxide solution. After neutralization with sulphuric acid and removal of the barium sulphate formed, the supernatant fluid was analysed chromatographically (butan-1-ol–acetic acid–water; 50:12:50, by vol.), and the amino acids, including monoiodotyrosine and di-iodotyrosine, were determined by ninhydrin colorimetry.

Methods

Immunization procedure. The antigens used were incorporated in a water in oil–adjuvant mixture, according to Salk & Laurent (1952). Ten parts of 5% gelatin-derivative solution, 1 part of Arlacel A (Atlas Powder Co., Wilmington, Del., U.S.A.) and 9 parts of Bayol F (Stanco Distributors, New York, U.S.A.) were homogenized by repeated filling and forcible ejection from a syringe. The adjuvant mixture contained 0·35 mg. of dead tubercle bacilli/ml.

Each material was injected into two rabbits. After pre-immunization bleedings, the antigen was administered into the thighs of the hind legs of the animals. Four injections of 0·5 ml. of the adjuvant mixture were given, at fortnightly intervals.

The animals were bled weekly by cardiac puncture, 2–5 weeks after the last injection. Thiomersal was added to all sera to a concentration of 0·01%.

Quantitative precipitin studies. In preliminary tests qualitative precipitin reactions were carried out on sera of individual rabbits. In all experiments the antisera to a particular gelatin derivative behaved similarly. Thereafter such antisera were pooled before quantitative studies.

(*a*) In all cases increasing amounts of antigen dissolved in 0·75 ml. of aqueous 0·85% sodium chloride were added to test tubes containing 0·5 ml. of serum. In control experiments aqueous 0·85% sodium chloride solution was added in the absence of antigen. The contents of the tubes were mixed, placed in a water bath at 37° for 1 hr., and then in the cold room for 24 hr. or, in some cases, for 48 hr. The tubes were centrifuged at 2° and, whenever precipitates were formed, they were washed three times with chilled (2°) aqueous 0·85% sodium chloride, dissolved in 0·1N-sodium hydroxide (1·5 ml.) and neutralized with N-hydrochloric acid. The extinction of these solutions was read at 280 mμ. The amount of antigen in the precipitate was determined from the hydroxyproline content of the acid hydrolysate (6N-hydrochloric acid; 105°, 24 hr.). The antibody content was obtained from the extinction after deducting the calculated extinction of the antigen.

(*b*) Where no precipitates were formed under the above-described conditions, precipitin reactions were carried out essentially according to Maurer (1954*a*). After being stored in the cold room for about 10 days, each serum sample was centrifuged in the cold and the lipid was removed from the top. The serum was decanted and recentrifuged until perfectly clear. Increasing amounts of antigen dissolved in 0·5 ml. of aqueous 0·85% sodium chloride solution were added to test tubes containing 3 ml. of serum. The tubes were mixed, capped, placed in a water bath at 37° for 1 hr. and kept at 2° for 12 days, with mixing of the tubes daily. The tubes were centrifuged and the precipitates formed were treated and analysed as described under method (*a*). Precipitates formed in control test tubes were similarly analysed and the results deducted from the values obtained.

Inhibition studies. Substances checked as possible inhibitors of the precipitin reactions were added at various concentrations, in a volume of 0·1 ml., to 0·2 ml. of serum. After an incubation of 30 min. at 37° an amount of antigen corresponding to the optimum precipitation was added, and the mixture was incubated at 37° for 1 hr. and then placed in the cold room for 24 hr.

Hydroxyproline. This was determined in the hydrolysates of the antigen–antibody precipitates, as well as in the hydrolysates of the gelatin derivatives themselves, by the method of Neuman & Logan (1950). Bone gelatin contains 13·3% of hydroxyproline (Eastoe, 1955).

Radioactivity. Radioactivity of antigen–antibody precipitates, as well as of the supernatant fluids, was measured in a well-type Tracerlab Scintillation Counter.

Spectrophotometric measurements. These were made on a Beckman model DU spectrophotometer, at approximately 25°, with quartz cells of 1 cm. light path. In the readings of solutions of antigen–antibody precipitates, cells with a capacity of 1 ml. were used.

RESULTS

Immunological response of polypeptidyl gelatins

All the polypeptidyl gelatins listed in Table 1 were tested for an immunological response. None of the materials gave any precipitate when added to pre-immunization sera. The antisera were subjected to the precipitin test. Positive reactions were obtained with polytyrosyl, polytryptophyl, polyphenylalanyl and polycysteinyl gelatins. The other gelatin derivatives, which gave no precipitations when examined by the usual methods, were tested by the more sensitive technique of Maurer (1954*a*).

Table 4. *Composition of precipitates of the system poly-L-tyrosyl gelatin–anti-poly-L-tyrosyl gelatin*

Antigen added (μg./ml. of serum)	Antigen precipitated, from hydroxyproline data* (μg.)	Antigen precipitated, from radioactivity data (μg.)	Antigen precipitated† (%)	Antibody precipitated‡ (μg.)	Antibody/antigen in precipitate (w/w)	Presence of antigen in the supernatant fluid	Presence of antibody in the supernatant fluid
50	52·0	49·6	100	210	4·2	−	+ +
100	73·7	56·2	65	268	4·85	−	+ +
250	95	92·5	37·5	369	3·94	−	+
500	95	97·0	19·2	481	5·02	±	−
750	83·4	81·4	11	481	5·84	+ +	−
1000	80	77·2	7·9	449	5·68	+ + +	−
1500	64·4	54·4	4	372	6·19	·	·

* Bone gelatin contains 13·3% of hydroxyproline (Eastoe, 1955).
† Calculated from the average of data obtained by the hydroxyproline and the radioactivity methods.
‡ Average of results of three experiments.

Polytyrosyl gelatin. Table 4 gives the analysis of the precipitates from the antiserum against poly-tyrosyl gelatin tested with the homologous antigen. In this case the amount of antigen in the precipitate was determined not only from its hydroxyproline content, but also by using labelled antigen. Because of the presence of tyrosine in pTyrGel it was possible to iodinate it with ^{131}I. The results obtained with both methods are in reasonable agreement. The amount of antibody was derived from the extinction of solutions of the precipitates at 280 mμ, after deducting the extinction of the antigen present. The precipitin curve is given in Fig. 1.

The antibodies to pTyrGel are very specific. Anti-pTyrGel was precipitated only by its homologous antigen, by other tyrosine derivatives of gelatin containing differing amounts of tyrosine (see the next paper, Arnon & Sela, 1960) and by pAzoTyrGel. No precipitation occurred when any of the following materials was added, at three different concentrations (20, 200 and 1000 μg.), to 0·2 ml. of the antiserum: gelatin, pTryGel, pPheGel, pGluGel, pLysGel, pAlaGel, egg albumin, poly-L-tyrosyl egg albumin, tyrosine, glycyl-L-tyrosine, tri-L-tyrosine as well as all of the copolymers containing tyrosine or di-iodotyrosine mentioned in Table 3.

All those materials that did not precipitate anti-pTyrGel were tested for their ability to inhibit the specific precipitin reaction. Table 5 summarizes the inhibition experiments for copolymers of amino acids, as well as poly-L-tyrosyl egg albumin and poly-L-tyrosyl edestin. As seen from Table 5, the inhibition was successful only for copolymers rich in tyrosine or polytyrosyl proteins. No inhibition was observed when tyrosine, trityrosine, gelatin, various polypeptidyl gelatins or polyglutamic acid was added.

Polytryptophyl gelatin. Table 6 gives the analysis of the precipitates from the antiserum against

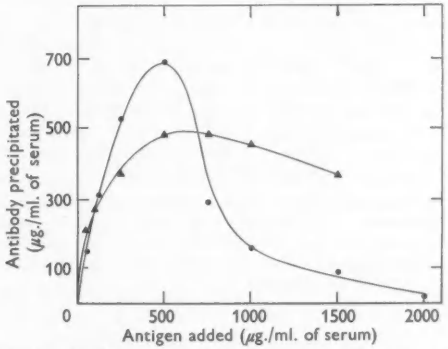

Fig. 1. Homologous precipitin curves of poly-L-tryptophyl gelatin (●) and poly-L-tyrosyl gelatin (▲).

Table 5. *Inhibition of poly-L-tyrosyl gelatin–anti-poly-L-tyrosyl gelatin precipitation*

Inhibitor	Amount added (μg./0·2 ml. of serum)	Precipitate
Poly-L-tyrosyl egg albumin	200	±
	1000	−
Poly-L-tyrosyl edestin	200	±
Copolymer Tyr.Glu (1:1)	50	+ + +
	200	±
	1000	−
Copolymer Tyr.Glu (1:4)	100	+ + +
	300	+ +
	2000	+
Copolymer Tyr.Glu (1:9)	150	+ + +
	500	+ + +
	3000	+ + +
Copolymer Tyr.Leu.Glu (1:1:1)	100	+ +
	250	+
	1000	±
Copolymer Tyr.Ala (1:10)	250	+ + +
	1000	+ + +
Iodinated Tyr.Glu copolymer	100	+ + +
	300	+
	1000	−

polytryptophyl gelatin, tested with the homologous antigen. The precipitin curve is given in Fig. 1. The antibodies to pTryGel are specific, similarly to the antibodies to pTyrGel. In addition to the homologous antigen, they could be precipitated only by poly-L-tryptophyl egg albumin. Fig. 2 gives the extinction of the solutions of the precipitates of poly-L-tryptophyl egg albumin–anti-pTryGel. A similar curve for pTryGel–anti-pTryGel is given for comparison.

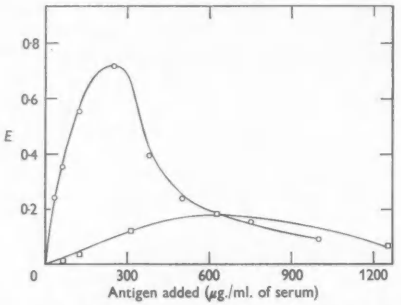

Fig. 2. Extinction of neutral solutions of precipitates, obtained by the addition of poly-L-tryptophyl gelatin (○) and poly-L-tryptophyl albumin (□) to the antiserum to poly-L-tryptophyl gelatin, as a function of the amount of the precipitant.

Polyphenylalanyl gelatin. The analysis of the precipitates from the antiserum against polyphenylalanyl gelatin tested with the homologous antigen is given in Table 6. In Fig. 3 is shown its precipitin curve. No precipitation of antibodies to pPheGel was observed when gelatin, egg albumin, pTyrGel, pGluGel, poly-L-phenylalanyl egg albumin, as well as all the copolymers containing phenylalanine mentioned in Table 3, were added. All these materials were tested subsequently for their ability to inhibit the precipitin reaction. As seen from Table 7, the only material that caused complete inhibition was a copolymer of L-phenylalanine and L-glutamic acid, in a residue molar ratio 1:0·87.

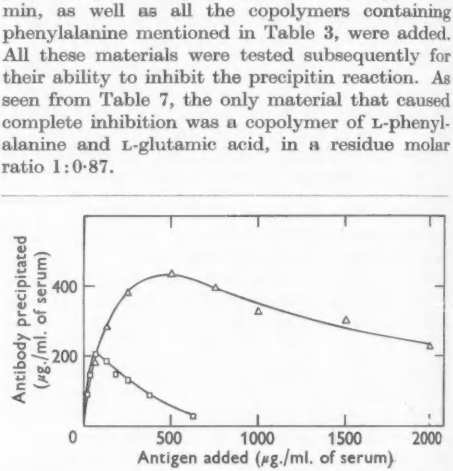

Fig. 3. Homologous precipitin curves of poly-L-phenylalanyl gelatin (△) and poly-L-cysteinyl gelatin (○).

Table 6. *Composition of precipitates of the systems pTryGel–anti-pTryGel, pPheGel–anti-pPheGel and pCysGel–anti-pCysGel*

Antigen	Antigen added (μg./ml. of serum)	Antigen precipitated (μg.)	Antigen precipitated (%)	Antibody precipitated (μg.)	Antibody/antigen in precipitate (w/w)
pTryGel	60	45·6	76	144	3·16
	125	49·8	40	308	6·2
	250	70·4	27	522	7·4
	500	91·2	18·5	668	7·3
	750	66·4	8·9	288	4·35
	1000	45·6	4·6	153	3·4
	1500	29·0	1·9	89	3·1
	2000	16·6	0·8	14·6	—
pPheGel	60	53	88·3	182	3·4
	125	72	57·5	284	3·9
	250	103	41·2	380	3·7
	500	127	25·4	434	3·5
	750	154	20·5	396	2·6
	1000	147	14·7	326	2·25
	1500	137	9·1	304	2·2
	2000	127	6·3	227	2·1
pCysGel	12·5	12·9	100	90	6·9
	31	30·5	98	147	4·8
	62·5	43·4	69·5	206	4·7
	125	39·7	31·7	183	4·6
	187·5	35·4	18·9	148	4·2
	250	31·8	12·7	132	4·15
	375	22·0	5·9	90	4·1
	625	4	0·6	29	—

Polycysteinyl gelatin. The composition of the precipitates of the pCysGel–anti-pCysGel system is given in Table 6. The precipitin curve is shown in Fig. 3. The form of the curve is similar to those obtained for pTyrGel, pTryGel and pPheGel, but the amount of the precipitated antibody is considerably smaller. No precipitation of antibodies to pCysGel was observed when gelatin, pTyrGel, pSerGel or egg albumin was added. When these materials were tested, only pSerGel, at 1000 μg., caused complete inhibition, whereas gelatin and pTyrGel, at 1000 μg., only partially inhibited the pCysGel–anti-pCysGel precipitation.

Other polypeptidyl gelatins. Antisera against pAlaGel, pGluGel and pLysGel, as well as against gelatin treated with anhydrous hydrogen bromide, gave no precipitates with the homologous antigens when subjected to the usual tests. Nor was any precipitation observed when the antisera were brought into cross-reaction with the various gelatin derivatives. The precipitin test was therefore performed in a way similar to that used by Maurer (1954*a*) to demonstrate the antigenicity of unmodified gelatin. Table 8 gives the amount of antibodies that could be precipitated by the homologous antigens in the equivalence zone. Maurer (1954*b*) found 78 μg. of antibody against unmodified gelatin in 1 ml. of rabbit antiserum. The data of Table 8 therefore indicate that the treatment with anhydrous hydrogen bromide did not alter the extent of antigenicity of gelatin. In view of this it may be concluded that any changes observed in the antigenicity of pTyrGel, pGluGel and pLysGel, as compared with gelatin, are due to the enrichment of gelatin with the respective amino acids and not to the treatment with anhydrous hydrogen bromide during their preparation.

The antibodies to polyglutamyl gelatin were also precipitated by gelatin, by multi-chain polyglutamic acid and by a gelatin derivative enriched both with tyrosine and glutamic acid (pGluTyrGel, see the next paper, Arnon & Sela, 1960). Precipitation curves for all the above are given in Fig. 4.

Table 7. *Inhibition of pPheGel–anti-pPheGel precipitation*

Inhibitor	Amount added (μg./0·2 ml. of serum)	Precipitate
Gelatin	50	+ + +
	200	+ +
	1000	+
Egg albumin	50	+ + +
	200	+ + +
	1000	+ + +
Poly-L-phenylalanyl egg albumin	50	+ + +
	200	+ +
	1000	+
pTyrGel	50	+ + +
	200	+ +
	1000	+
pGluGel	50	+ + +
	200	+ +
	1000	+
Copolymer Phe.Glu (1:1)	50	+ +
	200	+
	1000	−
Copolymer Phe.Glu.(1:2)	50	+ + +
	200	+ +
	1000	+
Copolymer Phe.Glu (1:4)	200	+ + +
	500	+ + +
	2000	+ + +

Non-precipitable antibodies

As seen from Tables 4, 6 and 8, the precipitates in the equivalence zone contain only 20–70 % of the total antigen added. In this region no precipitate occurs upon addition of either antigen or antibody to the supernatant fluid. This implies heterogeneity of antigen or of antibody. Gelatin is not a monodisperse substance, and its use as a multi-functional initiator in the polymerization of *N*-carboxy-α-amino acid anhydrides contributes, though in a limited way (Katchalski, Gehatia & Sela, 1955), to the polydispersity of the product. Nevertheless, the fact that at high antibody–antigen ratios all the antigen is found in the precipitate suggests that the antibodies to the various polypeptidyl gelatins are at least of two types, namely precipitating and non-precipitable.

To explore the possibility that the non-precipitable antibody in the antiserum against pTyrGel might be an antibody to unmodified gelatin, the following experiment was performed: A great excess of gelatin was added to antiserum against

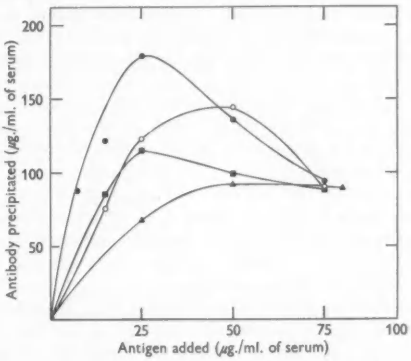

Fig. 4. Precipitin curves of poly-L-glutamyl gelatin (●), gelatin (■), multichain poly-L-glutamic acid (▲) and copoly-(L-glutamyl, L-tyrosyl)gelatin (○) with the antiserum to poly-L-glutamyl gelatin.

Table 8. *Antibodies precipitated by gelatin treated with hydrogen bromide, pAlaGel, pGluGel and pLysGel from the respective antisera*

Antigen	Antigen added (μg./ml. of serum)	Antigen precipitated (μg.)	Antigen precipitated (%)	Antibody precipitated (μg.)	Antibody/ antigen in precipitate (w/w)
Gelatin–HBr	15	—	—	32	—
	30	8	28	93	11·6
	50	—	—	57	—
pAlaGel	15	—	—	85	—
	30	20	67	192	9·6
	50	—	—	152	—
pGluGel	15	—	—	93	—
	30	19	63	183	9·6
	50	—	—	130	—
pLysGel	15	13	86	133	10·2
	30	—	—	174	—
	50	27	54	179	6·7

Table 9. *Amount of antibodies precipitated from 1 ml. of antiserum in the equivalence zone*

	Precipitate obtained by the usual technique		Precipitate obtained by Maurer's technique	
Antigen	Antigen added to cause maximal precipitation (μg.)	Antibody precipitated in the equivalence zone (μg.)	Antigen added to cause maximal precipitation (μg.)	Antibody precipitated in the equivalence zone (μg.)
pTyrGel	500	481	500	570
pTryGel	500	668	—	—
pPheGel	500	434	—	—
pCysGel	65	206	—	—
pLysGel	—	0	50	179
pGluGel	—	0	30	183
pAlaGel	—	0	30	192
Gelatin–HBr	—	0	30	93

pTyrGel. After incubation for 1 hr., an amount of pTyrGel that should give the maximal precipitation was added. The precipitate, composed of pTyrGel and precipitating antibody against pTyrGel, was removed, and the supernatant fluid was tested for the presence of free pTyrGel by addition of more antiserum. No precipitate was formed.

If the non-precipitable antibodies had been antibodies to gelatin, they would have been bound strongly by the gelatin added at the beginning of the experiment and would not have been available for pTyrGel. The fact that no free pTyrGel could be detected in the supernatant fluid suggests that the antiserum to pTyrGel indeed contains non-precipitable antibodies which are specific to pTyrGel and not to gelatin.

A parallel experiment with the pPheGel system yielded similar results. A more direct proof of the existence of a soluble antigen–antibody complex in the supernatant fluid at the equivalence zone, based on electrophoretic experiments with a radioactively labelled antigen, will be given in the next paper (Arnon & Sela, 1960).

Comparison of the various polypeptidyl gelatins

The results obtained with the various polypeptidyl derivatives of gelatin are collected in Table 9. With a good antigen, e.g. pTyrGel, the use of Maurer's technique increases only to a small extent the amount of antibody precipitated from the serum. On the other hand, poor antigens, which do not cause any precipitation under the usual conditions, are able in Maurer's technique to precipitate amounts of antibodies similar to those obtained, e.g. with pCysGel, by the usual techniques. Thus it seems that the criteria for the antigenicity of a protein should include not only the amount of antibodies that can be elicited by the antigen, but also the conditions under which these antibodies can be detected.

DISCUSSION

The experiments described demonstrate that enrichment of gelatin with α-amino acids containing aromatic structures in their side chain converts gelatin into relatively powerful antigens. It cannot

be decided yet if this enhancement results specifically from the aromatic character of the amino acids attached or if it is an example of a more general enhancement in antigenicity due to an increase in the rigidity of the molecule. Studies now in progress on the antigenicity of poly*cyclo*hexylalanyl gelatin will enable us to answer this question.

The attachment of cysteine residues to gelatin also contributed to an increase in antigenicity although to a much smaller degree. This, again, might be due to an increase in the rigidity of the protein. Even though the content of sulphydryl groups in pCysGel did not change significantly during a period of several months, it is possible that disulphide bonds, which would increase the rigidity of the macromolecule, are formed *in vivo*.

The very limited change in the antigenic potency of gelatin caused by enrichment with lysine or glutamic acid suggests that the polar amino acid residues have no significant enhancing ability although they may still exercise considerable influence on the specificity of antigens.

The antibodies to all the polypeptidyl gelatins possess a very narrow specificity, being precipitated almost exclusively by the homologous antigens. Only with anti-pTryGel was there cross-reaction with poly-L-tryptophyl egg albumin. It should be noticed that the enrichment with tryptophan was higher than in any other gelatin derivative.

In all cases we found specific inhibitors that could interfere with the precipitation of the antibodies by the homologous antigens. From the chemical structure of these inhibitors, information can be obtained about the size and character of the reacting site on the antibody molecule, as well as on the chemical nature of the immunologically active site on the antigen molecule. The pTyrGel and pPheGel systems could be inhibited by copolymers rich in tyrosine and phenylalanine respectively. Such copolymers possess a random sequence, i.e. they contain in their chains regions of repeating units of tyrosine or of phenylalanine. As copolymers with a lower ratio of the aromatic α-amino acid, where the chance of finding adjacent residues of tyrosine or phenylalanine in the chain is very small, have no inhibiting properties, it must be assumed that sequences of several tyrosine or phenylalanine residues are necessary for the inhibition.

The inhibition of a precipitin reaction involving one gelatin derivative, by various other modified gelatins, shows that gelatin contributes towards the specificity of the immunological reaction. Thus anti-pPheGel is inhibited not only by the pTyrGel (enriched with aromatic groups) but also by pGluGel and by gelatin itself. Moreover, with Maurer's precipitin technique, gelatin precipitated anti-pGluGel.

All the gelatin derivatives investigated gave rise to non-precipitable as well as precipitating antibodies. The presence of non-precipitable antibodies in immunological systems has been repeatedly reported. The techniques used were based mainly on the labelling of the antigen with ^{131}I (Burtin, 1955; Feinberg, 1958). However, it is conceivable that the persistence of radioactivity in supernatant fluids was due to inhomogeneous labelling, so that one small fraction of antigen is so highly labelled that it reacts as a heterologous cross-reacting antigen. An enzyme which is not completely inhibited by antibody permits the detection of such soluble complexes without any chemical modification of the antigen (Cinader, 1957).

The amount of the antigen in the precipitate and in the supernatant fluid was here calculated from the hydroxyproline content. This method has been used previously by Maurer (1954*a*), who showed, while studying the antigenicity of gelatin in man, that in the equivalence zone only 50% of the gelatin was present in the precipitate. The reasonably good agreement between the data obtained for pTyrGel with both the hydroxyproline and the radioactive-iodine techniques suggests that the danger of uneven isotopic labelling is small in these systems.

It was shown in this study that the attachment of some non-antigenic peptides to a poorly antigenic protein yields potent antigens. From various inhibition experiments it appears that gelatin is definitely involved in the immunologically active sites of these antigens. On the other hand, the peptide chains attached contribute strongly to the immune specificity. The role of the peptides and of gelatin in the specificity of polypeptidyl gelatins is discussed in detail in the next paper (Arnon & Sela, 1960).

SUMMARY

1. Several polypeptidyl derivatives of gelatin and albumin have been synthesized and characterized. They include polyalanyl, polyglutamyl, polylysyl, polycysteinyl, polytyrosyl, polytryptophyl and polyphenylalanyl gelatins. Various copolymers of α-amino acids have also been prepared and analysed.

2. The enrichment with tyrosine, tryptophan and phenylalanine converted gelatin into relatively powerful antigens, as followed by the precipitin reaction. The attachment of cysteine to gelatin caused a limited enhancement of antigenicity.

3. Antibodies to polyalanyl, polyglutamyl and polylysyl gelatins could not be detected by the usual precipitin techniques, but their presence could be shown by a method similar to that used by Maurer (1954*a*) to demonstrate the antigenicity of gelatin.

4. The antibodies to polypeptidyl gelatins could be precipitated almost exclusively by the homologous antigens. However, it was possible to interfere with the precipitin reactions by means of specific inhibitors. From the inhibition experiments it was concluded that the polypeptidyl chains, and in some cases also gelatin itself, contribute towards the specificity of the antigens.

5. The presence of non-precipitable as well as precipitating antibodies to all the gelatin derivatives investigated was shown.

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REFERENCES

Anfinsen, C. B., Sela, M. & Tritch, H. (1956). *Arch. Biochem. Biophys.* **65**, 156.
Arnon, R. & Sela, M. (1960). *Biochem. J.* **75**, 103.
Beaven, G. H. & Holiday, E. R. (1952). *Advanc. Protein Chem.* **7**, 319.
Becker, R. R. & Stahmann, M. A. (1953). *J. biol. Chem.* **204**, 745.
Ben-Ishai, D. & Berger, A. (1952). *J. org. Chem.* **17**, 1564.
Berger, A., Kurtz, J., Sadeh, T., Yaron, A., Arnon, R. & Lapidoth, Y. (1958). *Bull. Res. Counc. Israel*, **7A**, 98.
Berger, A., Noguchi, J. & Katchalski, E. (1956). *J. Amer. chem. Soc.* **78**, 4483.
Bergmann, M., Zervas, L. & Ross, W. F. (1935). *J. biol. Chem.* **111**, 245.
Boyd, W. C. (1956). *Fundamentals of Immunology*. New York: Interscience Publishers Inc.
Boyer, P. D. (1954). *J. Amer. chem. Soc.* **76**, 4331.
Burtin, P. (1955). *Bull. Soc. Chim. biol., Paris*, **37**, 977.
Cinader, B. (1957). *Annu. Rev. Microbiol.* **11**, 371.
Clutton, R. F., Harington, C. R. & Yuill, M. E. (1938). *Biochem. J.* **32**, 1111.
Eastoe, J. E. (1955). *Biochem. J.* **61**, 589.
Feinberg, R. (1958). *J. Immunol.* **81**, 14.
Go, Y. & Tani, H. (1939). *Bull. chem. Soc. Japan*, **14**, 510.
Goodwin, T. W. & Morton, R. A. (1946). *Biochem. J.* **40**, 628.
Harrington, W. F. & Sela, M. (1958). *Biochim. biophys. Acta*, **27**, 24.
Haurowitz, F. (1950). *Chemistry and Biology of Proteins*. New York: Academic Press Inc.
Humphrey, J. H. & Yuill, M. E. (1939). *Biochem. J.* **33**, 1826.
Katchalski, E. (1957). In *Methods in Enzymology*, vol. 3, p. 540. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Katchalski, E. & Berger, A. (1957). In *Methods in Enzymology*, vol. 3, p. 546. Ed. by Colowick S. P. & Kaplan, N. O. New York: Academic Press Inc.
Katchalski, E., Gehatia, M. & Sela, M. (1955). *J. Amer. chem. Soc.* **77**, 6175.
Katchalski, E. & Sela, M. (1953). *J. Amer. chem. Soc.* **75**, 5284.
Katchalski, E. & Sela, M. (1958). *Advanc. Protein Chem.* **13**, 243.
Kay, R. E., Harris, D. C. & Entenman, C. (1956). *Arch. Biochem. Biophys.* **63**, 14.
La Rosa, W. (1927). *Chem. Anal.* **16**, no. 2, 3.
Landsteiner, K. (1945). *The Specificity of Serological Reactions*. Cambridge, Mass.: Harvard University Press.
Landsteiner, K. & van der Scheer, J. (1939). *J. exp. Med.* **69**, 705.
Levy, A. L. (1954). *Nature, Lond.*, **174**, 126.
Makinodan, T., Becker, R. R., Wolfe, H. R. & Stahmann, M. A. (1954). *J. Immunol.* **73**, 159.
Maurer, P. H. (1954*a*). *J. exp. Med.* **100**, 497.
Maurer, P. H. (1954*b*). *J. exp. Med.* **100**, 515.
Maurer, P. H. (1955). *Arch. Biochem. Biophys.* **58**, 205.
Maurer, P. H. (1957). *Proc. Soc. exp. Biol., N.Y.*, **96**, 394.
Maurer, P. H., Subrahmanyam, D., Katchalski, E. & Blout, E. R. (1959). *J. Immunol.* **83**, 193.
Mechanic, G. L. & Levy, M. (1959). *J. Amer. chem. Soc.* **81**, 1889.
Micheel, F. & Schallenberg, E. (1952). *Hoppe-Seyl. Z.* **291**, 87.
Neuman, R. E. & Logan, M. A. (1950). *J. biol. Chem.* **184**, 299.
Neumann, H., Levin, Y., Berger, A. & Katchalski, E. (1959). *Biochem. J.* **73**, 33.
Patchornik, A., Sela, M. & Katchalski, E. (1954). *J. Amer. chem. Soc.* **76**, 299.
Salk, J. E. & Laurent, A. M. (1952). *J. exp. Med.* **95**, 429.
Sanger, F. (1952). *Advanc. Protein Chem.* **7**, 1.
Sela, M. (1954). *Bull. Res. Counc. Israel*, **4**, 109.
Sela, M. & Berger, A. (1955). *J. Amer. chem. Soc.* **77**, 1893.
Sela, M. & Haurowitz, F. (1958). *Experientia*, **14**, 91.
Sela, M. & Katchalski, E. (1955). *J. Amer. chem. Soc.* **77**, 3662.
Sela, M. & Katchalski, E. (1959). *Advanc. Protein Chem.* **14**, 391.
Sela, M., Katchalski, E. & Gehatia, M. (1956). *J. Amer. chem. Soc.* **78**, 746.
Sela, M., Katchalski, E. & Olitzki, A. L. (1956). *Science*, **123**, 1129.
Sela, M., White, F. H., jun. & Anfinsen, C. B. (1959). *Biochim. biophys. Acta*, **31**, 417.
Stahmann, M. A., Tsuyuki, H., Weinke, K., Lapresle, C. & Grabar, P. (1955). *C.R. Acad. Sci., Paris*, **241**, 1528.
Szent-Gyorgyi, A. G. & Cohen, C. (1957). *Science*, **126**, 696.
Talmage, D. W., Baker, H. R. & Akeson, W. (1954). *J. infect. Dis.* **94**, 199.
Tristram, G. R. (1953). In *The Proteins*, vol. 1, part A, p. 181. Ed. by Neurath, H. & Bailey, K. New York: Academic Press Inc.
Yaron, A. & Berger, A. (1958). *Bull. Res. Counc. Israel*, **7A**, 96.

Studies on the Chemical Basis of the Antigenicity of Proteins

2. ANTIGENIC SPECIFICITY OF POLYTYROSYL GELATINS

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In the preceding paper it was shown that the attachment of tyrosine, tryptophan or phenylalanine peptides to gelatin converts it into a relatively powerful antigen. The serological specificity of these gelatin derivatives varied according to the nature and the length of the peptides attached. Thus the specificity of polytyrosyl gelatin (10% of tyrosine residues) and polytryptophyl gelatin (25% of tryptophan residues) was due, respectively, to polytyrosine and polytryptophan chains, but not to gelatin. On the other hand, with polyphenylalanyl gelatin (9% of phenylalanine residues), it could be shown that the specificity is associated not only with the phenylalanine chains but also with gelatin itself.

In view of these findings it seemed of interest to enquire how the potency of the new antigens depends on the number of amino acids attached to gelatin. By enhancement or increase in antigenicity we understand an increase, relative to gelatin, in the eliciting of antibodies by these antigens, even if they are serologically different. It also seemed worthwhile to examine the change in the serological specificity of polypeptidyl gelatins as a function of the length of the polypeptides attached.

In this paper we report the results of an investigation of the extent of antigenicity and the antigenic specificity of a series of polytyrosyl gelatins differing in their tyrosine content. We have found that the attachment of 2% of tyrosine to gelatin suffices to convert it into a relatively strong antigen. Yet the specificity of this antigen is due mainly to gelatin.

In view of the assumed role of polar groups in the antigenic specificity of proteins, we have also investigated the antigenicity of gelatin enriched with copolymers of tyrosine and glutamic acid. Although the attachment of polar amino acid residues alone does not significantly enhance the antigenicity of gelatin (Sela & Arnon, 1960), glutamic acid in company with tyrosine affected both the extent and the specificity of the immunological response.

EXPERIMENTAL

Materials

Amino acids and peptides are abbreviated as in Sanger (1952) (see Sela & Arnon, 1960). The samples of gelatin, egg albumin, edestin, poly-L-glutamic acid and tri-L-tyrosine ethyl ester were described in the preceding paper (Sela & Arnon, 1960).

The polytyrosyl gelatins described below were prepared from gelatin and the corresponding *N*-carboxytyrosine anhydrides according to the method for the synthesis of polypeptidyl gelatins given previously (Sela & Arnon, 1960).

Poly-L-tyrosyl gelatin A (pTyrGel A). This was prepared by the reaction of *O*-benzyloxycarbonyl-*N*-carboxy-L-tyrosine anhydride with gelatin, followed by the removal of the benzyloxycarbonyl groups of the modified gelatin with anhydrous hydrogen bromide. This sample was denoted in the preceding paper as pTyrGel.

Poly-L-tyrosyl gelatin B (pTyrGel B) *and poly-L-tyrosyl gelatin C* (pTyrGel C). These were prepared by adding respectively 0·75 and 0·15 m-mole of *N*-carboxy-L-tyrosine anhydride (Berger *et al.* 1958)/g. of gelatin. In these cases unprotected carboxytyrosine anhydride was used, as it was found that, under the conditions of polymerization used, the phenolic hydroxyl groups do not interfere with the reaction. The water-soluble reaction products were dialysed against distilled water at 2° for 3 days and lyophilized.

Copoly-(L-glutamyl, L-tyrosyl) gelatin (pGluTyrGel). This (see Table 1) was prepared by adding a mixture of 1·5 m-mole of γ-benzyl *N*-carboxy-L-glutamate anhydride and 1 m-mole of *N*-carboxy-L-tyrosine anhydride/g. of gelatin. After the completion of the reaction 2 vol. of cold acetone were added, and the precipitated gelatin derivative was washed with acetone and dried *in vacuo* over phosphorus pentoxide. The benzyl groups were removed by the action of anhydrous hydrogen bromide in acetic acid at 2° for 65 hr. (Yaron & Berger, 1958). pGluTyrGel was precipitated with ether, dissolved in water at pH 7·0 (N-sodium hydroxide was added for the neutralization), dialysed against distilled water at 2° for 3 days and lyophilized.

Labelled pGluTyrGel. This was prepared by iodination of pGluTyrGel with ^{131}I (150 μc/10 mg.) according to Talmage, Baker & Akeson (1954). The substance isolated after exhaustive dialysis had a radioactivity of 700 000 counts/min./mg.

All the other polypeptidyl gelatins, polytyrosyl egg albumin and polytyrosyl edestin, as well as all the copolymers containing tyrosine, were described in the preceding paper (Sela & Arnon, 1960).

Methods

Amino acid analysis. The content of tyrosine in the various gelatin derivatives was calculated from the extinction of an alkaline solution (pH 13·0) at 293·5 mμ.

In order to determine the content of glutamic acid in pGluTyrGel, the gelatin derivative was hydrolysed with 6N-hydrochloric acid at 105° for 24 hr. The hydrolysate was subjected to paper electrophoresis on Whatman no. 1

filter paper in a phthalate buffer, 0·025 M, pH 5·92 (5·10 g. of potassium hydrogen phthalate and 0·86 g. of sodium hydroxide in 1 l. of water), at a potential gradient of 10 v/cm., at 25°, for 2 hr. It was then chromatographed in the second dimension in butanol–acetic acid–water (50:12:50, by vol.) for 48 hr. Glutamic acid was estimated by ninhydrin colorimetry (Kay, Harris & Entenman, 1956).

Hydroxyproline was determined in the hydrolysates (6 N-hydrochloric acid; 105°, 24 hr.) of the antigen–antibody precipitates, as well as in the hydrolysates of the gelatin derivatives themselves, by the method of Neuman & Logan (1950).

Dinitrophenylation. Dinitrophenylation of the various gelatin derivatives was carried out at pH 9·0 at 40° for 90 min. Dinitrophenyl (DNP)-tyrosine, DNP-glutamic acid and ε-*N*-DNP-lysine were determined colorimetrically after hydrolysis (Levy, 1954; Anfinsen, Sela & Tritch, 1956).

Immunization. Immunization procedures, quantitative precipitin tests and inhibition studies were performed as described in the preceding paper (Sela & Arnon, 1960).

Spectrophotometric measurements. These were made on a Beckman model DU spectrophotometer, at approx. 25°, with 1 cm. quartz cells. In the readings of solutions of antigen–antibody precipitates, cells with a capacity of 1 ml. were used.

Electrophoretic analyses. Analyses of the sera were made on Whatman no. 1 filter-paper strips in Michaelis buffer (*I* 0·1), pH 8·6, at a potential gradient 2 v/cm., at 25° for 17 hr.

RESULTS

The polytyrosyl gelatin derivatives were investigated for their antigenicity in rabbits. All antisera gave positive precipitin tests. Antibodies against the three polytyrosyl gelatins differing in their tyrosine content (pTyrGel A, pTyrGel B and pTyrGel C) could be cross-precipitated with all the compounds listed in Table 1. On the other hand, antibodies to the gelatin derivative containing both tyrosine and glutamic acid (pGluTyrGel) could be precipitated only by the homologous antigen. Additional information about the chemical nature of the immunologically active groups on the antigen molecules was obtained from a study of the inhibition of the specific precipitin reactions with the help of various protein derivatives and copolymers of α-amino acids.

Precipitin reactions with homologous antigens

Table 2 gives the analysis of the precipitates from the antisera against the polytyrosyl gelatin derivatives listed in Table 1, tested with the respective homologous antigens. The amount of antigen in the precipitate was calculated from its hydroxyproline content. The amount of antibody was obtained from the extinction of solutions of the precipitates at 280 mμ, after deducting the extinction of the antigen present. The data for pTyrGel A were reported in the preceding paper (pTyrGel, see Sela & Arnon, 1960), but are given here for comparison.

The maximal amounts of antibodies that could be precipitated from the antisera against the various polytyrosyl gelatins, as a function of their tyrosine content, are shown in Fig. 1. These results were obtained with the usual precipitin techniques, and under such conditions antibodies to gelatin were not precipitated. As seen from Fig. 1, there was almost no change in the extent of antigenicity as the enrichment of gelatin with tyrosine residues was lowered from 11 to 6 %. Gelatin enriched with 2·4 % of tyrosine residues still elicited a considerable amount of antibodies, though significantly less than the other polytyrosyl gelatins. Additional decrease in the tyrosine content seems to cause a steep decrease in antigenicity. Since only two animals were used in each experiment, the quantitative values of the differences must be

Table 1. *Polytyrosyl gelatins*

A	B	C	D	E	F	G
Gelatin derivative	Percentage of tyrosine (and glutamic acid) residues in the gelatin derivative*	Enrichment† (%)	Tyrosine (and glutamic acid) attached (moles/100 000 g. of gelatin)	Ratio of α-amino groups to ε-amino groups‡	Number of moles of distinct polypeptide chains/100 000 g. of gelatin§ [30·6 E/(E + 1)]	Average no. of amino acid residues per peptide chain attached (D/F)
pTyrGel A	10·3	11·1	68	2·35	21	3·2
pTyrGel B	5·7	5·7	35	2·85	23	1·5
pTyrGel C	2·7	2·4	15	0·79	14	1·1
pGluTyrGel Glu:	13·0	3·5	27	0·92‖	15	4·3
pGluTyrGel Tyr:	5·2	6·0	37			

* The original gelatin contains 0·31 % of Tyr and 10·15 % of Glu residues.

† Calculated, assuming gelatin as 100 %.

‡ Determined from the absorptions of the DNP-amino acids attached, and of ε-DNP-lysine, recovered from the hydrolysates of the dinitrophenylated derivatives.

§ Calculated, assuming 30·6 lysine residues/100 000 g. of gelatin (Eastoe, 1955).

‖ DNP-Tyr accounted for 77 moles % of the total α-amino content; DNP-Glu accounted for the other 23 moles %.

Table 2. *Composition of precipitates of the systems pTyrGel A–anti-pTyrGel A, pTyrGel B–anti-pTyrGel B, pTyrGel C–anti-pTyrGel C and pGluTyrGel–anti-pGluTyrGel*

Antigen	Antigen added (μg./ml. of serum)	Antigen precipitated (μg.)	Antigen precipitated (%)	Antibody precipitated (μg.)	Antibody/ antigen in precipitate (w/w)
pTyrGel A	50	52·0	100	210	4·2
	100	73·7	74	268	4·85
	250	95·0	37	369	3·94
	500	95·0	19	481	5·02
	750	83·4	11	481	5·84
	1000	80·0	7·9	449	5·68
	1500	64·4	4	372	6·19
pTyrGel B	60	51·0	85	392	7·7
	125	60·8	49	430	7·1
	250	67·2	27	470	7·0
	500	69·0	14	386	5·6
	750	67·2	9	266	3·95
	1000	44·0	4·4	212	4·8
	1500	—	—	76	—
pTyrGel C	10	11·3	100	110	9·7
	25	27·2	100	236	8·7
	50	41·5	83	262	5·9
	100	54·3	54·3	321	5·8
	150	64·3	43	296	4·6
	250	50·0	20	248	5·0
	500	45·8	9·2	155	3·4
pGluTyrGel	10	12·3	100	512	41
	20	22·4	100	624	28
	50	45	90	766	17
	100	70·6	71	684	9·6
	150	77·2	51	620	8·1
	200	77·2	38·6	596	7·7
	300	70·6	23·5	492	7·0
	500	45	9	282	6·2
	1000	6·5	0·7	42	6·4

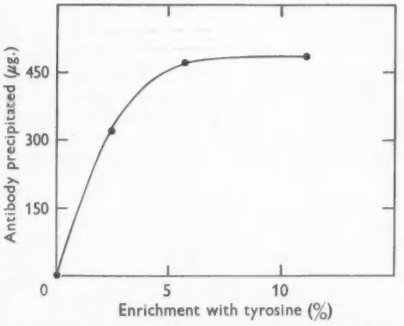

Fig. 1. Maximal amounts of antibodies precipitated in 1 ml. of serum, from the antisera against the polytyrosyl gelatins included in Table 1 and against gelatin, as a function of the extent of the enrichment of gelatin with tyrosine residues.

taken with some reservation. Qualitatively, however, the differences are quite clear.

pGluTyrGel is the most powerful among the antigens investigated (Table 2). This derivative, in which gelatin was enriched with 6·0 % of tyrosine residues as well as 3·5 % of glutamic acid residues, precipitated in the equivalence zone 766 μg. of antibodies from 1 ml. of the corresponding antiserum. This should be compared with the maximal precipitation of 470 μg. of antibodies against pTyrGel B by the homologous antigen, in which gelatin was enriched with 5·7 % of tyrosine residues exclusively.

Cross-precipitations

All the polytyrosyl gelatin derivatives investigated yielded precipitates when added to the antiserum against pTyrGel A. Fig. 2 shows the extinctions of the solutions of these precipitates as a function of the amount of the gelatin derivative added. As the tyrosine content of the antigen is lowered, less and less antibodies are precipitated in the equivalence zones, and higher amounts of the antigens are needed to cause maximal precipitation. pGluTyrGel and pTyrGel B contain almost the same percentage of tyrosine and it seems therefore of interest that they give very similar precipitin curves.

Fig. 3 gives the precipitin curves of the various polytyrosyl derivatives of gelatin with the antiserum against pTyrGel B. The amount of the anti-

bodies precipitated by the homologous antigen is somewhat higher than the amounts obtained by precipitation with the other antigens. Either an increase or a decrease in the tyrosine content of the precipitant caused a decrease in the extent of the precipitation. The differences between the various precipitin curves are smaller in this case than in that of either anti-pTyrGel A (Fig. 2) or anti-pTyrGel C (Fig. 4). This is not surprising, because the maximal difference in tyrosine content between pTyrGel B and any other polytyrosyl gelatin derivative is smaller than for pTyrGel A and pTyrGel C.

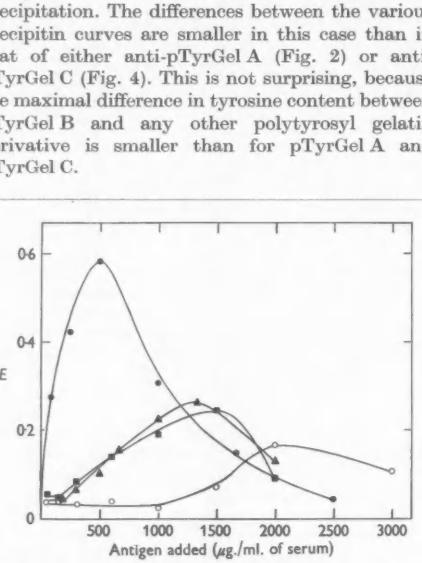

Fig. 2. Extinction, at 280 mμ, of neutral solutions of precipitates (dissolved in 0·1 N-sodium hydroxide and neutralized with N-hydrochloric acid) obtained by the addition of pTyrGel A (●), pTyrGel B (■), pTyrGel C (○) and pGluTyrGel (▲) to the antiserum to pTyrGel A, as a function of the amount of the precipitant.

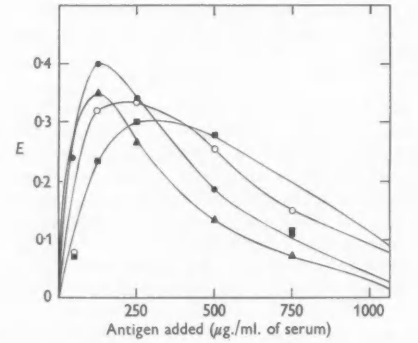

Fig. 3. Extinction, at 280 mμ, of neutral solutions of precipitates obtained by the addition of pTyrGel A (○), pTyrGel B (●), pTyrGel C (■) and pGluTyrGeL (▲) to the antiserum to pTyrGel B, as a function of the amount of the precipitant.

Anti-pTyrGel C could be precipitated not only by all the four polytyrosyl gelatins investigated, but also by unmodified gelatin (Fig. 4), as well as by gelatin enriched with alanine, glutamic acid, lysine, cysteine, phenylalanine or tryptophan. The homologous antigen was again the best precipitant. These findings demonstrate that gelatin, rather than the polytyrosine chains, determines the specificity of pTyrGel C. The fact that gelatin is a

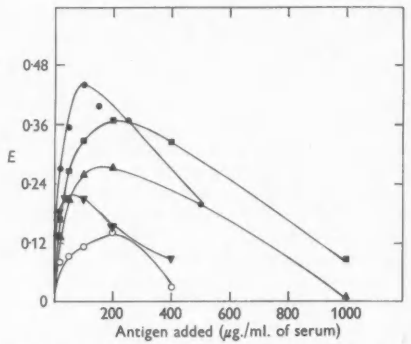

Fig. 4. Extinction, at 280 mμ, of neutral solutions of precipitates obtained by the addition of pTyrGel A (○), pTyrGel B (■), pTyrGel C (●), pGluTyrGel (▲) and gelatin (▼) to the antiserum to pTyrGel C, as a function of the amount of the precipitant.

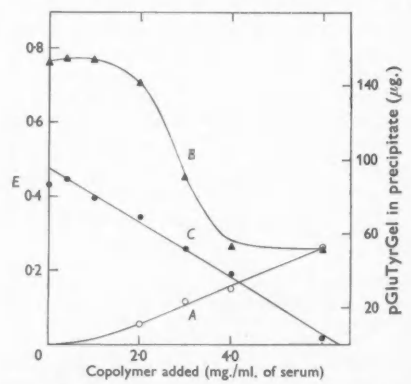

Fig. 5. *A*, Extinction of neutral solutions of precipitates obtained by the addition of a copolymer of L-tyrosine and L-glutamic acid, in a residue molar ratio 1:1, to the antiserum to pGluTyrGel, as a function of the amount of the copolymer. *B*, Extinction of neutral solutions of precipitates obtained by the addition of the homologous antigen (50 μg.) to mixtures of antiserum to pGluTyrGel (1 ml.) and increasing amounts of the copolymer. *C*, Amount of pGluTyrGel in the precipitates shown in curve *B*, calculated from their hydroxyproline content.

better precipitant of the antibodies to pTyrGel C than is pTyrGel A shows that the presence of short tyrosine chains does not significantly influence the antigenic specificity of gelatin.

In contrast with the three polytyrosyl gelatins, the gelatin derivative containing both tyrosine and glutamic acid elicited antibodies which were precipitated only by the homologous antigen. These antibodies were not precipitated by polyglutamyl gelatin, by any of the polytyrosyl gelatins or by mixtures of polyglutamyl gelatin or polyglutamic acid with the various polytyrosyl gelatins. The only other substance which caused precipitation of the antibodies to pGluTyrGel was a copolymer of L-tyrosine and L-glutamic acid in a residue molar ratio 1:1 (curve *A* in Fig. 5). The precipitation occurs only at high concentrations of the copolymer.

No precipitation occurred when any of the following substances was added to each of the four antisera investigated: egg albumin, polytyrosyl egg albumin, tyrosine, trityrosine, copolymers of L-tyrosine and L-glutamic acid in residue molar ratios of 1:4 and 1:9, and a copolymer of L-tyrosine, L-leucine and L-glutamic acid in a residue molar ratio of 1:1:1. No precipitation occurred when gelatin, unmodified or enriched with various amino acids, was added to pTyrGel A, pTyrGel B or pGluTyrGel, nor when a copolymer of L-tyrosine and L-glutamic acid in a residue molar ratio 1:1 was added to the three polytyrosyl gelatins.

Inhibition studies

The results of studying the inhibition of the specific precipitin reaction with various reagents are given in Table 3.

The change in the antigenic specificity as the tyrosine content of the gelatin derivatives is decreased is illustrated in the inhibition experiments with the copolymers of L-tyrosine and L-glutamic acid in a molar ratio of 1:1, and of L-tyrosine, L-leucine and L-glutamic acid in a molar ratio of 1:1:1. Both copolymers are efficient inhibitors of the systems in which the antigen is rich in tyrosine but have almost no effect on the precipitation of pTyrGel C–anti-pTyrGel C, where the antigen is enriched with only 2·4% of tyrosine residues. pTyrGel A and pTyrGel B contain tyrosine as peptide sequences, whereas in pTyrGel C tyrosine is attached to the amino groups of gelatin as a monomeric unit. On the other hand, the copolymers possess a random sequence, i.e. they may contain in their chains, to some extent, regions of repeating units of tyrosine. The copolymer of L-tyrosine and L-glutamic acid in molar ratio of 1:4 is a more effective inhibitor of the pTyrGel B–anti-pTyrGel B system than of the systems in which the antigen is either richer or poorer in tyrosine. The copolymer of Tyr:Glu, molar ratio 1:9, in which the chance of tyrosine peptide sequences is extremely small, does not significantly inhibit any of the precipitin reactions investigated. It seems that tyrosine peptide chains

Table 3. *Inhibition tests*

In all cases 50, 200 and 1000 μg. quantities of the inhibiting substance were added to 0·2 ml. of each antiserum, 30 min before the addition of the homologous antigen. −, Complete inhibition even at the lowest inhibitor concentration; ±, complete inhibition at the highest inhibitor concentration, inhibitory influence even at the lowest concentration; +, partial inhibition at the highest concentration, no effect at the lowest concentration; ++, only slight inhibition at the highest concentration; +++, no inhibitory effect.

Inhibitor	Effect on pTyrGel A system	Effect on pTyrGel B system	Effect on pTyrGel C system
Poly-L-tyrosyl egg albumin	−	+	+
Poly-L-tyrosyl edestin	−	.	++
Copolymer Tyr:Glu (1:1)	±	+	++
Copolymer Tyr:Glu (1:4)	+	±	++
Copolymer Tyr:Glu (1:9)	+++	++	++
Copolymer Tyr:Leu:Glu (1:1:1)	±	+	++
Copolymer Tyr:Ala (1:10)	+++	.	.
Iodinated copolymer Tyr:Glu (1:1)	±	.	.
Trityrosine	+++	+++	+++
Gelatin	+++	±	−*
pPheGel	+++	+	−*
pGluGel	+++	+	−*
pTryGel	+++	+	−*
pAlaGel	+++	.	−*

* 10, 20, 50, 100, 200 and 500 μg. quantities of the inhibitory substance were added to 0·5 ml. of antiserum. Amounts up to 200 μg. caused partial precipitation. The precipitates were centrifuged off, and the homologous antigen was added to the supernatant fluids. pTyrGel C–anti-pTyrGel C precipitates were obtained up to 50 μg. of the inhibitor, only traces of the specific precipitates were formed at 100 μg. of the inhibitor and complete inhibition occurred at 200 and 500 μg. of the inhibitor.

are involved in the antigenic specificity of pTyrGel A and pTyrGel B. The inhibitory effect of polytyrosyl egg albumin and polytyrosyl edestin is in agreement with this conclusion. The inability of trityrosine to inhibit the precipitin reactions suggests that the tyrosine peptide sequences must form a part of a macromolecule in order to be effective.

Neither unmodified gelatin nor gelatin enriched with amino acids other than tyrosine inhibited the pTyrGel A–anti-pTyrGel A system. They caused only a partial inhibition of the pTyrGel B–anti-pTyrGel B system, and were very effective with the pTyrGel C–anti-pTyrGel C system, both as precipitants and inhibitors. It may be concluded from these experiments that the antigenic specificity of pTyrGel C, and to some extent of pTyrGel B, is associated with the gelatin moiety.

With the pGluTyrGel system none of the following substances had any inhibitory effect: gelatin, polyglutamyl gelatin, the three polytyrosyl gelatins and mixtures of polyglutamyl gelatin, polyglutamic acid or glutamic acid with the various polytyrosyl gelatins or tyrosine. All the copolymers listed in Table 3 were also tested for their influence on this system. Only the copolymer of Tyr:Glu (1:1) was effective, as an inhibitor at lower concentrations and as a precipitant at higher concentrations (Fig. 5). Curve *A* of Fig. 5 shows the precipitation of the antibodies to pGluTyrGel with the copolymer. Curve *B* shows that the amount of precipitate formed upon addition of the homologous antigen, half an hour after the addition of the copolymer, decreases with the increase in the concentration of the copolymer. At copolymer levels high enough to cause considerable precipitation of anti-pGluTyrGel, addition of the homologous antigen did not increase the amount of the precipitate. The inhibitory effect of the copolymer is demonstrated also in curve *C*, which shows the decrease in the homologous antigen content of the precipitates as a function of the copolymer concentration. It may be concluded therefore that the copolymer indeed reacts specifically with the antibodies to pGluTyrGel.

Non-precipitable antibodies

With all the gelatin derivatives investigated, only a part of the antigen added was found in the precipitates in the equivalence zone. As at high antibody–antigen ratios all the antigen was present in the precipitate, it was concluded that the antigen not found in the precipitate in the equivalence zone is bound to non-precipitable antibodies (Sela & Arnon, 1960). The existence of this soluble complex in the supernatant fluid could not be demonstrated by electrophoretic separation, as the polypeptidyl gelatins migrated close to the γ-globulins under the conditions used for the resolution of the serum components. Only with pGluTyrGel could the presence of non-precipitable antibodies be shown unequivocally.

^{131}I-labelled pGluTyrGel was used for this experiment. Quantities of 20, 70 and 300 μg. were added to 1 ml. of the anti-pGluTyrGel serum. The precipitates formed after 48 hr. were centrifuged off and the supernatant fluids were subjected to electrophoresis on paper strips. A control experiment, performed with a mixture of rabbit normal serum (1 ml.) with 300 μg. of the labelled antigen, was performed. After drying of the paper strips, they were radioautographed for 16 days on Kodak AA film.

No radioactivity was found in the supernatant fluid from the antibody-excess zone (20 μg. of antigen/ml. of serum). In the supernatant fluid from the equivalence zone (70 μg. of antigen/ml. of serum) almost all of the radioactivity was found in the γ-globulin region, and only traces of the free antigen, which migrates between α- and β-globulins, were present. In the antigen-excess region (300 μg. of antigen/ml. of serum) one large black stain appeared on the radioautogram, covering the zones both of the γ-globulin and of the free antigen. In the control experiment no radioactivity was found in the area of the γ-globulins; the blackening occurred only in the region occupied by the free antigen. Thus the antigen investigated does not react with normal serum to give non-specific complexes that would migrate with γ-globulin. It may be concluded therefore that the soluble complex formed by pGluTyrGel and γ-globulins results from the interaction of the antigen with the specific antibodies.

DISCUSSION

It may be concluded from the experiments described that the extent of the antigenicity of the gelatin derivatives depends on the amount of aromatic α-amino acid attached.

At the same time the serological specificity of the gelatin derivatives changes strongly with variations in the tyrosine content. Cross-precipitation and inhibition experiments demonstrate that the specificity of the antigens is determined by the length of the peptide chains attached to gelatin. Thus the antigenic specificity of pTyrGel A resides exclusively in the tyrosine peptide chains. With pTyrGel B both gelatin and compounds containing tyrosine had an effect on the specific precipitin reaction, whereas with pTyrGel C almost all the serological specificity is due to the gelatin moiety. The attachment of monomeric units of tyrosine to half the amino groups of gelatin caused a strong enhancement in antigenicity, essentially without changing the specificity.

Whereas the attachment of polar amino acid residues to gelatin caused almost no change in the extent of antigenicity (Sela & Arnon, 1960), the

enrichment of gelatin with both tyrosine and glutamic acid yielded an antigen that elicited more antibodies than a polytyrosyl gelatin with a similar tyrosine content. The antibodies to pGluTyrGel are specific. This is in agreement with the common assumption that the polar groups strongly influence the antigenic specificity of proteins. The only substance that interacted with, and even precipitated, these antibodies was a copolymer of glutamic acid and tyrosine, thus indicating that all the antigenic specificity lies in the peptide chains attached to gelatin.

Electrophoretic experiments have shown that the antigen which is not precipitated in the equivalence zone is bound to antibodies in the form of a soluble complex. This proves the existence of both precipitating and non-precipitable antibodies in the antisera to gelatin and its various derivatives.

It appears from this investigation that the attachment of a very limited amount of tyrosine residues enhances dramatically the antigenicity of gelatin, without essentially changing its antigenic specificity. This may serve indirectly as an additional proof of the antigenicity of unmodified gelatin. On the other hand, an increase in the amount of tyrosine, or tyrosine and glutamic acid, bound to gelatin results in changes not only in the extent of antigenicity but also in the serological specificity of the gelatin derivatives obtained. The question arises in these cases whether gelatin still contributes in any way towards the immunological properties of the new antigens. An answer to this question may come from a study of the antigenic properties of synthetic molecules composed of peptides or copolymers, but containing no protein moiety.

SUMMARY

1. Three polytyrosyl gelatins differing in their tyrosine content, as well as a gelatin enriched with both tyrosine and glutamic acid, were synthesized and tested for their antigenicity in rabbits. All antisera gave positive precipitin tests.

2. The extent of the antigenicity of the gelatin derivatives investigated depends on the amount of tyrosine attached. As little as 2% of tyrosine suffices to enhance strongly the antigenicity of gelatin.

3. The serological specificity of the gelatin derivatives changes strongly as a function of their tyrosine content. The specificity of compounds rich in tyrosine resides almost exclusively in the peptide chains attached, whereas it resides essentially only in the gelatin moiety in the derivative containing only 2% of tyrosine residues.

4. The attachment to gelatin of the polar glutamic acid in addition to tyrosine yielded an antigen of very narrow specificity.

5. Electrophoretic experiments have shown that the antigen which is not precipitated in the equivalence zone is bound to non-precipitable antibodies in the form of a soluble complex.

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REFERENCES

Anfinsen, C. B., Sela, M. & Tritch, H. (1956). *Arch. Biochem. Biophys.* **65**, 156.
Berger, A., Kurtz, J., Sadeh, T., Yaron, A., Arnon, R. & Lapidoth, Y. (1958). *Bull. Res. Counc. Israel*, **7A**, 98.
Eastoe, J. E. (1955). *Biochem. J.* **61**, 589.
Kay, R. E., Harris, D. C. & Entenman, C. (1956). *Arch. Biochem. Biophys.* **63**, 14.
Levy, A. L. (1954). *Nature, Lond.*, **174**, 126.
Neuman, R. E. & Logan, M. A. (1950). *J. biol. Chem.* **184**, 299.
Sanger, F. (1952). *Advanc. Protein Chem.* **7**, 1.
Sela, M. & Arnon, R. (1960). *Biochem. J.* **75**, 91.
Talmage, D. W., Baker, H. R. & Akeson, W. (1954). *J. infect. Dis.* **94**, 199.
Yaron, A. & Berger, A. (1958). *Bull. Res. Counc. Israel*, **7A**, 96.

Biochem. J. (1960) **75**, 109

Chromogenic Groupings in the Lowry Protein Determination

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Folin & Ciocalteu (1927), extending earlier work by Wu (1922), showed that a complex reagent containing molybdate, tungstate and phosphoric acid would react with proteins to yield a blue colour approximately proportional to the tyrosine and tryptophan content. Pressman (1943) defined some of the variables influencing colour yield. Herriott (1941) discovered that pretreatment with cupric ion greatly intensified the blue colour given by proteins and peptides, but had no effect upon the colour yield from tyrosine. Lowry, Rosebrough, Farr & Randall (1951) further refined and systematized these observations into a practical method of protein assay which, because of its

simplicity and sensitivity, has been widely adopted. Yet the molecular basis of the colour production has remained fairly obscure.

Our purpose was to investigate the nature of the chromogenic groups in proteins, and the mechanism whereby they reduce the phenol reagent in the presence of copper. It was found that whereas any peptide bond yields some colour, certain amino acid sequences are far more chromogenic than others, and these largely account for the colour yield of a protein. A substance presumed to be an oxidized product was obtained in small yield after reaction of histidylhistidine with the Lowry reagent; some of its properties and behaviour suggested a plausible mechanism of copper-catalysed electron transfer from dipeptide to phenol reagent. Finally, some of the conclusions derived from studies with simple peptides were shown to be valid for the B chain of insulin and its peptic-hydrolysis products.

EXPERIMENTAL

Colour reaction. This was performed as described by Lowry *et al.* (1951). Each lot of Folin–Ciocalteu phenol reagent (Braun–Knecht–Heimann, San Francisco) was tested as described by Oyama & Eagle (1956) to ascertain the optimum dilution. A barely visible amount of phenol red was added to each sample, which was then neutralized before the addition of reagents. To 1 ml. of sample was then added 5 ml. of Lowry's alkaline copper solution (in some experiments, as noted, copper was omitted). After 5–10 min. 0·5 ml. of properly diluted phenol reagent was jetted in. The blue colour was read 30 min. later in the Klett–Summerson colorimeter with red (no. 66) filter. A Klett unit (K.u.) is the amount of chromogenic material in 6·5 ml. of final reaction mixture which gives a Klett reading of unity. Data presented here have been corrected for reagent blanks (usual Klett reading 15–20). The term 'colour increment' is used to denote colour yielded by peptides and proteins in excess of that attributable to their component amino acids.

General procedures. Peptides and proteins were hydrolysed completely in 6N-HCl (sealed tubes) at 100° for 20 hr. and recovered by drying *in vacuo* over P_2O_5 and NaOH. A ninhydrin spray was used to locate amino acids or peptides on paper after chromatography or electrophoresis; narrow strips were sprayed and used as guides for elution of bands. The Pauly reaction was used to follow the imidazole ring of histidine on paper (Brown, Sanger & Kitai, 1955) or in solution (Macpherson, 1942). L-Cysteinylglycine and L-cysteinyl-L-valine were prepared through the benzyloxycarbonyl intermediates, as described by du Vigneaud & Miller (1952); identities were established by paper chromatography of the compounds and their hydrolysis products.

Paper electrophoresis was carried out in the Spinco–Durrum apparatus on strips of Whatman 3MM paper or on 31 cm. squares of such paper when large amounts of material were to be separated. The runs were at 13 v/cm. in collidine–acetic acid buffer (Lockhart & Abraham, 1954); to 9 ml. of γ-collidine were added 1 ml. of acetic acid and water to 1 l., pH 7·8 (Beckman model G pH meter). The duration of runs was varied to achieve optimum separation of bands in each case but was usually 2 hr. Bands were eluted with 0·1N-HCl and dried as described above.

For separation and identification of amino acids from hydrolysates, two-dimensional paper chromatograms were done on 22 cm. squares of Whatman 3MM paper, ascending technique, 3·5 hr. Solvents were ethanol–butanol–water–propionic acid (10:10:5:2, by vol.) and butanol–acetone–water–dicyclohexylamine (10:10:5:2, by vol.). Papers were sprayed with 0·25% solution of ninhydrin in acetone containing 7% of acetic acid, and developed for 3 min. at 75°. The characteristic colours and positions of the various amino acids described by Hardy, Holland & Nayler (1955) were confirmed in standard runs.

Isolation of an oxidized reaction product. The Lowry reaction was carried out with 30 mg. of His.His and all reagent volumes were 100 times greater than usual. The resulting 650 ml. of blue solution was poured onto a Dowex-50 (H) column (2·6 cm. × 50 cm.) prepared according to Moore & Stein (1951). The run-through (containing all the blue colour), the water-wash (300 ml.) and an aq. 0·1N-NH_3 soln. eluate (100 ml.) gave no Pauly reaction and were discarded. When the eluting solution was changed to aq. 1N-NH_3 soln., two fractions were obtained: an acidic fraction (20 ml.) and an alkaline fraction (100 ml.). Both fractions gave positive Pauly reactions. Each was dried *in vacuo*, subjected to paper electrophoresis at pH 7·8 and further prepared as described in the text.

Experiments with the B chain of insulin. Crystalline ox insulin was split into its two chains by Sanger's (1949) performic acid procedure. Chain B (50 mg.) was then dissolved in 25 ml. of 0·01N-HCl containing 2 mg. of pepsin (Nutritional Biochemicals Corp.). After 24 hr. at 37° the mixture was heated to 100° for 2 min., an equal volume of 10% trichloroacetic acid was added and, after standing overnight at 5°, the precipitate was removed by centrifuging. The supernatant was extracted four times with equal volumes of anhydrous ether and the digested products were recovered by lyophilization. Ten peptide bands were separated by paper electrophoresis at pH 7·8. After elution, a portion of each was tested for colour yield directly, and also after complete acid hydrolysis. A portion of the hydrolysate was also subjected to two-dimensional paper chromatography for identification of its component amino acids. The amino acid composition and the known points of cleavage by pepsin (Neurath, 1957) allowed us to assign unambiguous structures to nearly every peptide. The colour increment of each peptide was given by the colour yield before and after hydrolysis, but the exact amount of each peptide was not ascertained.

Abbreviations. For definition of the abbreviations used for amino acids in this paper see *Biochem. J.* (1957), **66**, 6.

RESULTS

Molecular configuration and colour yield. Preliminary complete hydrolysis reduced the colour-yielding property of albumin by more than two-thirds, and that of insulin by more than one-half (Table 1). Of the twenty common L-amino acids, only tyrosine, tryptophan, cysteine (and cystine) and, to a lesser extent, histidine gave significant

Table 1. *Effect of complete hydrolysis on colour yield of proteins*

The colour reaction was performed as described in the Experimental section.

		Crystalline* bovine albumin	Insulin†
Amount of protein	μg.	62·3	47·0
	μm-mole	0·904	8·20
Klett reading initially		103	96
K.u./μmole		114 000	11 700
K.u./μg.		1·65	2·04
Klett reading after complete hydrolysis		29	40
K.u./μmole		32 000	4 880
Colour remaining (%)		27	42

* Armour Laboratories, Kankakee, Ill. (mol.wt. taken as 69 000).

† Gift from Eli Lilly and Co., Indianapolis, Indiana (mol.wt. taken as 5733).

Table 2. *Colour yields of amino acids*

The colour reaction was performed as described in the Experimental section.

Amino acid	Amount (μmoles)	Klett reading	K.u./μmole
Tyrosine	0·1	126	1260
Tryptophan	0·1	112	1120
Cystine	1·0	128	128
Cysteine	2·0	122	61
Histidine	10·0	149	15
All others	5·0	<35	<7

colour (Table 2). Amino acid mixtures gave strictly additive colour values. D-Amino acids had the same colour yields as the L-isomers. The expected colour for the amino acids of the albumin hydrolysate, calculated from the data of Moore & Stein (1949), is 31 600 K.u./μmole; that from an insulin hydrolysate, based on the data of Ryle, Sanger, Smith & Kitai (1955), is 5450 K.u./μmole. The experimentally determined hydrolysate colours (Table 1) were in good accord with these expectations. It follows that the component amino acids account for but a fraction of the colour yield of a protein in the Lowry determination.

Data on the colour yields of peptides (Table 3) may be summarized as follows. All dipeptides developed some colour, even when their constituent amino acids did not. The colour increment due to a single peptide bond increased systematically with side-chain length in the series glycine, alanine, valine, leucine. Functional side chains (serine, threonine, methionine, asparagine) caused marked colour increments in both the glycine and alanine series. On the other hand, tyrosine and tryptophan, which yield considerable colour by themselves, underwent suppression of colour yield when linked to glycine or leucine. Cysteine dipeptides gave about the expected colour for cysteine alone. Dipeptides of histidine showed consistent colour increments. Tripeptides containing glycine or leucine gave very high colour increments, but one containing cysteine (glutathione) did not. Glycine tetrapeptide yielded much less colour than the tripeptide but still very much more than the dipeptide.

Table 3. *Colour yields of some peptides*

The colour reaction was performed as described in the Experimental section.

Peptide*	K.u./μmole: Found	K.u./μmole: Calc. from sum of amino acids
Dipeptides with *N*-terminal glycine		
Gly.Gly	14	0
Gly.DL-Ala	22	0
Gly.L-Val	26	0
Gly.DL-Val	28	0
Gly.L-Leu	40	0
Gly.DL-Leu	30	0
Gly.DL-Ser	65	3
Gly.DL-Thr	160	5
Gly.DL-Met	60	2
Gly.L-Asp–NH_2	270	6
Gly.D-Asp–NH_2	276	6
Gly.L-Tyr	990	1260
Gly.L-Try	676	1120
Dipeptides with *C*-terminal glycine		
Gly.Gly	14	0
DL-Ala.Gly	20	0
L-Leu.Gly	26	0
D-Leu.Gly	23	0
DL-Leu.Gly	21	0
L-CySH.Gly†	78	61
Dipeptides with *N*-terminal alanine		
DL-Ala.DL-Ala	40	0
DL-Ala.DL-Val	50	0
DL-Ala.L-Leu	6	0
DL-Ala.DL-Ser	14	3
DL-Ala.DL-Met	78	2
DL-Ala.L-Phe	28	4
DL-Ala.D-Asp–NH_2	329	6
DL-Ala.DL-Asp–NH_2	324	6
Other peptides		
L-CySH.L-Val†	64	61
L-His.Gly‡	138	12
L-His.L-Ala§	200	12
L-His.L-Leu§	256	12
L-His.L-His	760	24
L-Leu.L-Tyr	1090	1260
D-Leu.L-Tyr	960	1260
Gly.Gly	14	0
Gly.Gly.Gly	455	0
Gly.Gly.Gly.Gly	169	0
L-Leu.Gly.Gly	490	0
γ-L-Glu.L-CySH.Gly (glutathione)	76	61

* Unless otherwise noted, peptides were obtained from Nutritional Biochemicals Corp.

† These compounds were prepared in this Laboratory.

‡ Kindly supplied by Dr Emil L. Smith.

§ Kindly supplied by Dr Robert W. Holley.

A limited number of histidine dipeptides was available with blocked terminal groups. The data (Table 4) indicate that blocking the free amino group abolished the high colour increment but left an amount of colour approximately that to be expected from an ordinary peptide bond. Additionally blocking the free carboxyl group had no effect, but we lack information on histidine dipeptides blocked in this position alone. An important special case was that of His.His, the colour of which was reduced by blocking the terminal groups but still greatly exceeded the sum of colour yields from two histidine residues and the peptide bond.

Penicillin may be regarded as a curiously cyclized Cys.Val with a blocked terminal amino group. It yielded considerably more colour than Cys.Val (Table 4). After cleavage of the internal peptide bond by penicillinase (leaving the two amino acids linked through the cysteine S), the colour unexpectedly increased. Since desthiopenicillin gave no colour, the S must be essential to the reaction. Finally, penicillamine ($\beta\beta$-dimethylcysteine) yielded less colour than cysteine alone.

When the colour reaction was carried out in the absence of copper, as in the original Folin method, compounds fell into three categories (Table 5). Category I includes all peptides which yield more colour than their component amino acids and with these peptides copper was indispensable for the colour increment. Cystine, and to a lesser extent histidine, also depended upon copper for the colour they produced. Category II comprises peptides of tyrosine and tryptophan. These gave no colour increments. Tyrosine peptides yielded somewhat less colour than tyrosine alone and the colour was largely unaffected by copper. The only tryptophan peptide examined gave considerably less colour than the free amino acid and still less in the absence of copper. Category III consists of cysteine and its peptides, which gave much less colour in the presence of copper than in its absence.

Finally, the blue reaction product was found to have the same spectrum ($\lambda_{max.}$ 745, $\lambda_{min.}$ 405) for

Table 4. *Colour yields of some peptide derivatives*

The colour reaction was performed as described in the Experimental section. Cbz-, the benzyloxycarbonyl derivative. Enzymic hydrolysis of penicillin: 40 mg. of penicillinase (Nutritional Biochemicals Corp.) was incubated with 20 μmoles of penicillin-G sodium at pH 7·0, at 37° for 1 hr., followed by treatment with cold 5% trichloroacetic acid and colour determination on the supernatant. It was shown in an assay with *Sarcina lutea* that this procedure destroyed more than 99% of the antibacterial potency of the penicillin. Desthiopenicillin-G: this compound was prepared from penicillin-G with Raney nickel catalyst and recrystallized to constant melting point (105°) (Kaczka & Folkers, 1949).

Compound	K.u./μmole
Cbz-L-His.L-Ala*	32
(L-His.L-Ala)	200
Cbz-L-His.Gly ethyl ester†	38
(L-His.Gly)	138
Cbz-L-His.L-Leu methyl ester*	26
(L-His.L-Leu)	256
Cbz-L-His.L-His methyl ester†	208
(L-His.L-His)	760
Cbz-L-His.Gly.Gly ethyl ester†	306
Penicillin-G sodium	412
(L-CySH.L-Val)	64
Penicillinase-treated penicillin	580
Desthiopenicillin	0
Penicillamine HCl	16

* Kindly supplied by Dr Robert W. Holley.
† Kindly supplied by Dr Emil L. Smith.

Table 5. *Influence of copper on colour yield*

The colour reaction was performed as described in the Experimental section, except that special Lowry reagent was prepared without copper.

	Compound	K.u./μmole With Cu	K.u./μmole Without Cu	Difference (%)
I.	Crystalline bovine albumin	134 000	26 600	−80
	L-His.L-His	760	37	−95
	Gly.Gly.Gly	455	0	−100
	Penicillin-G sodium	412	10	−98
	L-Cystine	128	3	−97
	L-Histidine	15	4	−73
II.	L-Leu.L-Tyr	920	970	+5
	Gly.L-Tyr	990	980	−1
	L-Tyrosine	1 320	1 210	−8
	Gly.L-Try	676	368	−46
	L-Tryptophan	1 190	1 030	−13
III.	γ-L-Glu.L-CySH.Gly (glutathione)	62	700	+1 030
	$\beta\beta$-Dimethylcysteine (penicillamine)	16	256	+1 500
	L-Cysteine	61	194	+218

all types of reactant (amino acid, dipeptide, protein, Na_2SO_3), as would be expected if the final common step were reduction of the phenol reagent.

Nature of the oxidized product after reaction of His.His. We sought to isolate an oxidized product of the reaction of His.His with copper and phenol reagent as described under Methods. The principal component of the reaction mixture appeared in the alkaline eluate and was evidently unchanged His.His. It was identified by comparison with authentic His.His in paper electrophoresis and two-dimensional chromatography, by electrophoretic and chromatographic behaviour of its single hydrolysis product and by its ability to yield colour again in the Lowry reaction. The amount of unreacted peptide thus recovered was as great as 95% of the starting material in some experiments. Thus the colour yielded by His.His arises from oxidation of a very small fraction of the peptide initially present.

The only other component of the reaction mixture which gave a positive Pauly reaction for imidazole represented about 1% of the original His.His. This compound was eluted from the Dowex-50 (H) column in the acidic fraction, it gave a negative ninhydrin reaction and its electrophoretic migration was 6·0 cm. toward the anode in 2 hr. at pH 7·8. The Pauly colour of the band was typically pink, in contrast with the muddy brown obtained from an equimolar mixture of copper and His.His. Moreover, a direct H_2S test on the unknown band was negative, although copper in a ratio of 1 mole/imidazole group would have been detected readily. Evidently the unknown compound is not a copper complex. Its early elution from Dowex-50 (H), its negative ninhydrin reaction and its net negative charge at pH 7·8 indicate that the primary amino group has been removed or blocked.

The unknown band was eluted from paper, hydrolysed with 6N-HCl (100°, 20 hr.) and dried and subjected again to electrophoresis under the same conditions. Two bands were now obtained. One was identified as histidine. The other reacted with ninhydrin, but not with the Pauly reagent, and it migrated 8·8 cm. toward the cathode (cf. histidine 1·6 cm., His.His 1·3 cm.). Thus the unknown hydrolysis product appears to lack the imidazole ring, possesses a free amino group and has acquired a stronger net positive charge than histidine at pH 7·8.

Further studies of the presumed oxidized product were not undertaken and it would be premature to suggest any structure. However, the apparent absence of a free amino group in the unknown, and the recovery of intact histidine as a hydrolysis product, suggest that oxidation of His.His in the Lowry reaction may involve the removal of an electron pair from the primary amino group and imidazole ring of the *N*-terminal histidine residue.

Colour yields of peptides derived from insulin. When crystalline ox insulin was oxidized with performic acid to obtain chains A and B, there was a loss of about one-third of the total colour yield. By comparing the colour obtained after complete

Table 6. *Colour yields and colour increments of peptides obtained by peptic hydrolysis of chain B of insulin*

The colour reaction was performed as described in the Experimental section. The 10 bands are from paper electrophoresis of a peptic hydrolysate of insulin chain B.

Band	Total K.u.*	Percentage of total K.u.	Total K.u.* after complete hydrolysis	Percentage of colour remaining	Peptide structure†
1	210	0·6	0	0	Val.Glu
2	3 440	9·9	45	1·3	Val.Glu.Ala
3	1 160	3·3	50	4·3	Val.Glu.Ala.Leu
4	1 000	2·9	155	15·5	Leu.Val.Cy-SO_3H.Gly.Glu.Arg.Gly
5	3 940	11·4	375	9·5	Leu.Val.Cy-SO_3H.Gly.Glu.Arg.Gly.Phe
6	1 920	5·5	370	19·3	Tyr.Leu.Val.Cy-SO_3H.Gly.Glu.Arg.Gly.Phe
7	1 760	5·1	400	22·7	Val.Asp.Glu.His - - - - - - - - Glu.Arg.Gly.Phe‡
8	11 440	33·0	3 665	32·0	Indistinguishable from band 7‡
9	1 840	5·3	360	19·6	Val.Asp.Glu.His.Leu.Cy-SO_3H.Gly.Ser.His.Leu
10	7 950	23·0	4 020	50·4	Tyr.Thr.Pro.Lys.Ala
	34 660	100·0	9 440	27·2	—
K.u./μmole	4 820	—	1 310	—	—

* For material obtained from 7·19 μmoles of insulin B, initial colour yield 4540 K.u./μmole.

† Deduced from amino acid composition, expected points of cleavage and known sequences according to Ryle, Sanger, Smith & Kitai (1955).

‡ Bands 7 and 8 appear to be practically the whole chain less the terminal sequence of band 10. Phe is present in both but which of the three Phe residues are present in each cannot be stated.

hydrolysis of insulin with that found after complete hydrolysis of chains A and B it could be shown that part of the colour loss in the performic acid procedure was due to splitting of disulphide bridges and oxidation of cysteine residues to cysteic acid. Another part of the colour loss was due to partial oxidation of tyrosine residues.

The peptic digestion of chain B caused no further loss of chromogenicity. Paper electrophoresis yielded ten peptide bands whose colour yields and colour increments could be related to their amino acid sequences. The data (Table 6) show that the sum of the colour yields of the peptides was in good agreement with the colour yield of intact chain B. Peptic hydrolysis did not diminish chromogenicity because none of the points of cleavage is in a chromogenic sequence. Splitting at one of the histidine residues might well have caused a considerable colour loss.

The column of the table showing percentage of colour remaining after complete hydrolysis supports the following generalizations: (1) Sequences of the type Val.Glu, Val.Glu.Ala and Val.Glu.Ala.Leu give very large colour increments, although their contribution to the total colour yield of the protein is probably small. (2) Certain other sequences not represented among the synthetic peptides examined earlier are obviously chromogenic. In bands 4, 5 and 6, where the colour increments are considerable, we would suspect the Glu.Arg.Gly. The greater colour increment of band 5 than of band 4 implicates Gly.Phe as chromogenic. (3) The relatively high colour increment of band 9 can be attributed to its two sequences containing histidine. (4) The colour increment of band 10 shows that the sequence Thr.Pro.Lys.Ala must be chromogenic. This was confirmed by partial acid hydrolysis of band 10 (6N-HCl, 37°, 3 days), isolation of the tetrapeptide and determination of its colour yield before and after complete hydrolysis. (5) When tyrosine occurs in a large peptide (as in band 6) it reduces the colour increment (cf. band 5), as expected, but does not make a major contribution to the total colour. In a small peptide, on the other hand (as in band 10), tyrosine accounts for a much greater fraction of the total colour and the colour increment becomes correspondingly much smaller. (6) Finally, the residual colour yield of chain B after complete hydrolysis is accounted for almost entirely by its two tyrosine residues, as indicated by the total colour remaining in bands 6, 7, 8 and 10.

DISCUSSION

Tyrosine, tryptophan, cysteine and, to a very minor extent, histidine are capable of reducing the phenol reagent directly. Copper has little effect upon the colour yields of tyrosine and tryptophan but suppresses the reactivity of cysteine and its peptides, presumably by blockade of the sulphydryl group. Cystine produces colour only in the presence of copper. Any dipeptide yields some colour, provided that copper is present and the terminal amino group is free. The colour is greatly intensified by the presence of a side-chain nitrogen or carboxyl group on at least one of the component amino acids. Tripeptides are highly chromogenic even in the absence of functional side chains.

As to the mechanism of colour production, our observations suggest the following hypothesis, based upon the known capacity of peptides to form copper chelates (Martell & Calvin, 1952, pp. 525–531). In a dipeptide, the copper can be held in a coplanar tridentate chelate involving the free amino nitrogen, the peptide-bond nitrogen and the free carboxyl group. The additional presence of a side-chain nitrogen (as in asparagine or histidine) permits formation of a more stable, quadridentate chelate. In a tripeptide, a quadridentate chelate can be formed even in the absence of a functional side-chain group because the nitrogen atom of the second peptide bond is now available for co-ordination to copper. Chelation may be expected to facilitate electron removal from the peptide, as, for example, in the oxidation of ascorbic acid and the action of copper oxidases (Martell & Calvin, 1952, pp. 387–392).

As demonstrated for His.His, the colour yield does not represent complete oxidation of substrate, but rather the resultant of two simultaneous processes: the copper-catalysed electron removal from substrate, and the rapid destruction of electron acceptor in alkaline solution. Since the phenol reagent has a half-life of only 8 sec. at the alkaline pH of the reaction (Lowry *et al.* 1951), the rate of electron transfer will obviously be critical in determining the total colour produced. Our findings suggest that the tightness of binding of copper, especially the ability to form a quadridentate chelate, determines the rate of electron removal from substrate.

In the specific case of His.His our attempts to determine the structure of an oxidized product were inconclusive, but the partial information obtained is consistent with the reaction mechanism postulated above. Molecular models (Stuart-Briegleb models, Arthur S. LaPine Co., Chicago) demonstrated that copper can be co-ordinated in a quadridentate chelate to the free amino nitrogen, the peptide nitrogen, and a nitrogen atom of each imidazole ring. Evidence has been presented which suggests that an electron is then donated by the primary amino nitrogen, but the source of the second electron is uncertain.

Complete hydrolysis of a protein decreases its chromogenicity by one-half to two-thirds, and the

residual colour is almost wholly accounted for by its tyrosine and tryptophan content. Thus the major part of the colour yielded by proteins must arise from chromogenic amino acid sequences. It had seemed possible, at the outset of this investigation, that the large colour increments of proteins might be ascribed to a rather restricted set of sequences. Had that proved to be the case, the Lowry determination might conceivably have been used specifically to detect such sequences in polypeptides. However, the results with chain B of insulin, as well as those with simple dipeptides, point to the existence of a considerable number of different chromogenic sequences in proteins.

SUMMARY

1. In the Lowry protein determination the phenol reagent is reduced to yield a blue colour. The colour yield of a completely hydrolysed protein is due to its content of tyrosine, tryptophan and cysteine, the only amino acids that react significantly.

2. Preliminary treatment with alkaline copper solution leads to a great increase in the colour yield of proteins. We have shown with simple peptides as well as with chain B of insulin that this large colour increment is largely attributable to sequences of amino acids containing functional side chains. Particularly chromogenic are dipeptides containing a histidine, arginine or glutamic acid residue, the nature of the second residue being relatively unimportant.

3. The behaviour of a compound obtained in small yield and presumed to be the oxidized product of His.His suggests that electron removal from the dipeptide to the phenol reagent involves the amino nitrogen and imidazole ring of the *N*-terminal residue.

4. Efficient copper catalysis of electron transfer from a chromogenic grouping in protein to the phenol reagent is believed to require formation of a coplanar quadridentate chelate. The colour yield depends critically upon the rate of electron transfer since the phenol reagent is rapidly destroyed in the alkaline solution.

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REFERENCES

Brown, H., Sanger, F. & Kitai, R. (1955). *Biochem. J.* **60**, 556.
du Vigneaud, V. & Miller, G. (1952). *Biochem. Prep.* **2**, 74.
Folin, O. & Ciocalteu, V. (1927). *J. biol. Chem.* **73**, 627.
Hardy, T. L., Holland, D. O. & Nayler, J. H. C. (1955). *Analyt. Chem.* **27**, 971.
Herriott, R. M. (1941). *Proc. Soc. exp. Biol., N.Y.*, **46**, 642.
Kaczka, E. & Folkers, K. (1949). *The Chemistry of Penicillin*, p. 257. Ed. by Clarke, H. T., Johnson, J. R. & Robinson, R. Princeton, U.S.A.: University Press.
Lockhart, I. M. & Abraham, E. P. (1954). *Biochem. J.* **58**, 633.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
Macpherson, H. T. (1942). *Biochem. J.* **36**, 59.
Martell, A. E. & Calvin, M. (1952). *Chemistry of the Metal Chelate Compounds*. New York: Prentice-Hall.
Moore, S. & Stein, W. H. (1949). *J. biol. Chem.* **178**, 79.
Moore, S. & Stein, W. H. (1951). *J. biol. Chem.* **192**, 663.
Neurath, H. (1957). *Ann. N.Y. Acad. Sci.* **68**, 11.
Oyama, V. I. & Eagle, H. (1956). *Proc. Soc. exp. Biol., N.Y.*, **91**, 305.
Pressman, D. (1943). *Industr. Engng Chem. (Anal.)*, **15**, 357.
Ryle, A. P., Sanger, F., Smith, L. F. & Kitai, R. (1955). *Biochem. J.* **60**, 541.
Sanger, F. (1949). *Biochem. J.* **44**, 126.
Wu, H. (1922). *J. biol. Chem.* **51**, 33.

Biochem. J. (1960) **75**, 115

A Titrimetric Method for the Determination of Creatine Phosphokinase

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Creatine phosphokinase is usually assayed by measuring the amount of creatine or creatine phosphate formed in a standard time period or the initial rate of formation of one of these products (Askonas, 1951; Banga, 1943; Chappell & Perry, 1954; Ennor & Rosenberg, 1954; Kuby, Noda & Lardy, 1954; Narayanaswami, 1952; Rosenberg & Ennor, 1955). Oliver (1955) has described a method based on the coupling of adenosine triphosphate formation with reduction of triphosphopyridine

nucleotide; he points out that methods based on estimation of creatine or creatine phosphate are subject to errors arising from the introduction of the reactants with tissue extracts. Oliver's method has obvious disadvantages when inhibitor studies are contemplated, since two other enzymes are used in the system.

The method described below depends upon the fact that a hydrogen ion is quantitatively liberated at pH 9·0 in the reaction between adenosine triphosphate^{4-} and creatine^{-} to give adenosine diphosphate^{3-} and creatine phosphate^{2-} (Fiske & Subbarow 1929; Kumler & Eiler, 1943; Meyerhof & Lohmann, 1928; Noda, Kuby & Lardy, 1954). A constant pH titration can therefore be conducted, the rate of addition of base being equal to the rate of creatine ~P formation. The sensitivity is such that extreme dilutions of enzyme can be assayed with great accuracy in a few minutes. In the absence of substrates other than creatine and adenosine triphosphate a significant pH shift is produced only by hydrolysis of adenosine triphosphate or transphosphorylation of creatine; the former can be allowed for by a blank conducted without creatine and has proved to be very small in practice. The method is simple, rapid and easy to carry out; the results have proved to be reliable and reproducible.

EXPERIMENTAL

Creatine phosphokinase was prepared by procedure A of Kuby *et al.* (1954), but was not crystallized. Crystalline disodium adenosine triphosphate (ATP) was obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A. All other reagents used were of analytical grade.

A Beckman model GS pH meter was employed, the high-sensitivity range being used. A sleeve-type reference electrode was found to give greater stability and was used throughout the study. The temperature was maintained at 30° by means of a water-jacketed reaction vessel. The contents were stirred by a Teflon (polytetrafluoroethylene)-covered stirring bar and magnetic stirrer. A Gilmont Ultramicroburette of 0·1 ml. total capacity (Emil Greiner Co., New York, U.S.A.) and calibrated accuracy of $\pm 10^{-5}$ ml. was used, and the titration was conducted with carbonate-free 0·1387 N-NaOH. Since the total reaction volume was 20 ml., it remained essentially constant throughout the titration.

Procedure

The reaction mixture consisted of 10 ml. of 0·06 M-creatine (final concn. 0·03 M), 4 ml. of 0·02 M-ATP (final concn. 4 mM), 4 ml. of 0·01 M-manganous acetate (final concn. 2 mM), 1 ml. of 0·02 M-mercaptoacetic acid (final concn. 1 mM) and 1 ml. of creatine phosphokinase solution with an enzymic activity of less than 1·5 μmoles min.$^{-1}$ of creatine ~P. In experiments involving other concentrations and activators, the final volume was always made up to 20 ml. All components except the enzyme were placed in the reaction vessel and the pH was adjusted to 9·00 with 1 N-NaOH. The enzyme (at pH 9·0) was then introduced and base was added continuously from the burette to maintain a constant pH. Burette readings were noted at intervals of 1 min. For purposes of assay, the titration was continued for 5–10 min., depending upon the accuracy required. In the kinetic studies which were conducted to evaluate the optimum conditions for the assay procedure, the titration was continued for 25 min. or more. The stability of the pH meter was such that no significant error ($< 0{\cdot}1\%$) was introduced from this source.

Analysis of data

The slope of the titration curve (μmoles of base/min.) may be conveniently estimated by fitting a straight line by the method of least squares. When the interval on the abscissa is constant, as in this case, the slope of the line per unit interval is given by the following expressions (cf. Fisher & Yates, 1953), which may be rapidly evaluated without the necessity of graphical analysis.

$$\frac{2(y_5 - y_1) + (y_4 - y_2)}{10} \text{ (5 min. titration)},$$

$$\frac{3(y_7 - y_1) + 2(y_6 - y_2) + (y_5 - y_3)}{28} \text{ (7 min. titration)},$$

$$\frac{4(y_9 - y_1) + 3(y_8 - y_2) + 2(y_7 - y_3) + (y_6 - y_4)}{60} \text{ (9 min. titration)},$$

where y_i is the burette reading at i min.

RESULTS

Some initial experiments were carried out in which no thiol was incorporated into the reaction mixture. It was found that the reaction rate fell quite rapidly with time, closely approximating the apparent second-order kinetics with respect to ATP described under similar conditions by Kuby *et al.* (1954) (Fig. 1). After about 15 min., the titration

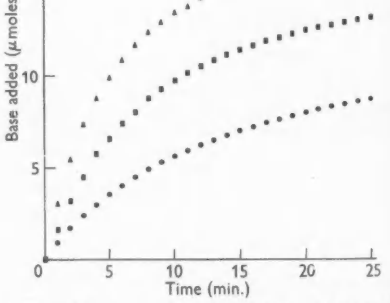

Fig. 1. Titration curves with 1 (●), 2 (■) and 3 (▲) ml. of enzyme preparation to system containing 1 mM-ATP, 6 mM-Mg^{2+} ion, 30 mM-creatine. Final volume, 20 ml.; pH 9·00.

curves obtained with different amounts of enzyme became almost parallel; the horizontal asymptote was evidently a function of the amount of enzyme added, implying that the enzyme was inactivated during the titration period. This possibility was confirmed by allowing the enzyme to stand in the reaction vessel with Mn^{2+} ions and ATP for 20 min. before addition of the creatine, whereupon no reaction occurred. Spontaneous inactivation of highly diluted creatine phosphokinase has previously been shown to be prevented by cysteine (Chappell & Perry, 1954; Oliver, 1955), and several thiols and other agents have therefore been examined for a similar protecting effect on this system. Fig. 2 shows representative titration curves obtained with the most effective of these. The rate falls much more slowly than in the control, and for the first 5–10 min. it is essentially constant. Larger concentrations (up to 10 mM) of the agents tested gave no better protection except with cysteine; in most cases some inhibition was observed. On the other hand, 2-mercaptoethanol or mercaptoacetate was equally effective at mM or 0·1 mM. Mercaptoacetate at 0·01 mM was only partially effective, but the reaction rate was better maintained than in its absence. A concentration of 0·5 mM-KCN was required for maximum effect. Sulphide, 2:3-dimercaptopropanol, ethylenediaminetetra-acetic acid and ascorbate were not effective as protecting agents at 1 mM; the first two inhibited the enzyme. Gelatin showed incomplete protection at a concentration of 1 mg./ml. The loss of activity which occurs in the absence of a protecting agent is reversible; the introduction of mercaptoacetate after the rate had decayed caused an immediate increase in rate to the value observed under standard conditions (Fig. 3).

The influence of varying the metal ion used to activate the system was examined. Mn^{2+} ion was found to give the maximum rate, followed by Mg^{2+} and Ca^{2+} ions (Fig. 4). The titration curves shown

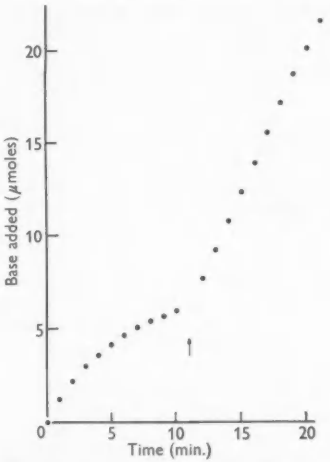

Fig. 3. Titration curve showing recovery of reaction rate on addition of sodium mercaptoacetate at arrow (final concn., 1 mM). Initial reactants were 4 mM-ATP, 2 mM-Mn^{2+} ion, 30 mM-creatine. Enzyme concentration was 0·30 μg. of N/ml. Final volume, 20 ml.; pH 9·00.

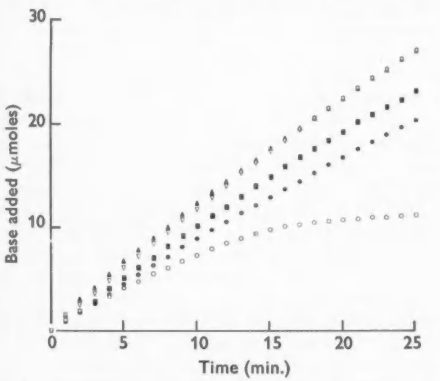

Fig. 2. Titration curves obtained with various protecting agents. 4 mM-ATP, 2 mM-Mn^{2+} ion, 30 mM-creatine. Enzyme concentration was 0·23 μg. of N/ml. Final volume, 20 ml.; pH 9·00. ○, Control; ■, 1 mM-cysteine; ▲, 1 mM-mercaptoethanol; ▽, 1 mM-mercaptoacetic acid; ●, 1 mM-KCN.

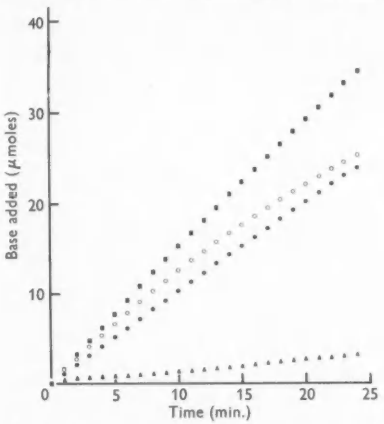

Fig. 4. Titration curves showing ▲, system without metal ions and activation by ●, Ca^{2+} (2 mM), ○, Mg^{2+} (4 mM) and ■, Mn^{2+} (2 mM) ions; 4 mM-ATP, 30 mM-creatine, 1 mM-mercaptoacetate. Enzyme concentration was 0·23 μg. of N/ml. Final volume, 20 ml.; pH 9·00.

in Fig. 4 were obtained with optimum concentrations of metal ions; Mn^{2+} and Ca^{2+} ions produced marked inhibition in higher concentrations, whereas Mg^{2+} ion was less effective in this respect. Fe^{2+} and Co^{2+} ions inhibited the enzyme. The optimum concentration was to some extent dependent on the concentration of mercaptoacetate used; when the latter was increased from 1 to 10 mM, the optimum concentration of Mn^{2+} ion increased from 2 to 4 mM. If the Mn^{2+} ion concentration exceeded the sum of the ATP and mercaptoacetate concentrations, the latter lost its protecting effect and a precipitate formed (presumably manganese hydroxide).

The optimum concentration of ATP was 4 mM, larger concentrations producing some inhibition of the enzyme. Smaller concentrations gave a satisfactory Lineweaver–Burk plot when $[Mn^{2+}]/[ATP]$ was kept at 0·5, with a Michaelis constant of 0·2 mM. Although the initial rate is relatively insensitive to ATP concentration in the range 1–4 mM, it is desirable to use the optimum concentration in the assay procedure to minimize the slight fall in rate which occurs as the reaction proceeds.

The titrimetric method is not suitable for studying the influence of pH on the reaction rate below pH 9·0, since the stoicheiometry of H^+-ion liberation fails as the pK_a values of the reactants are approached. However, there is no evidence of a sharp pH optimum, the rates at pH 8·5 and 9·5 being within 5 % of the rate at pH 9·0. Since the rate of spontaneous hydrolysis of ATP increases quite rapidly above pH 9·0, this pH is a suitable compromise for assay purposes.

With the standard conditions described in the Experimental section, the rate of H^+-ion liberation was linearly related to the amount of enzyme added (Fig. 5). The standard deviation of each estimation was $2{\cdot}6 \times 10^{-3}$ μmoles/min., which is well below 1 % of the range.

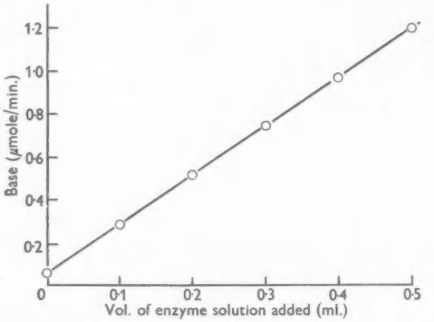

Fig. 5. Relation between reaction rate and amount of enzyme solution (containing 9·3 μg. of N/ml.) added. Duration of titration, 9 min.; 4 mM-ATP, 2 mM-Mn^{2+} ion, 1 mM-mercaptoacetate, 30 mM-creatine. Final volume, 20 ml.; pH 9·00.

Direct comparison with the initial velocity of creatine phosphate production by the method of Kuby *et al.* (1954) was made. The rate was only 55 % of that found by the titrimetric method described here. The addition of 1 mM-mercaptoacetate raised this to 65 %, and substitution of 2 mM-Mn^{2+} ion for 4 mM-Mg^{2+} ion further increased it to 70 %. The remaining difference is perhaps attributable to the glycine buffer used by Kuby *et al.* (1954); a direct comparison of the rate under identical conditions was impossible, since whereas the use of a strong buffer is mandatory with their technique it makes a constant-pH titration too insensitive.

The titrimetric method was found suitable for the estimation of creatine phosphokinase activity in a crude extract of rabbit muscle (10 mM-KCl) in an amount equivalent to 0·2–2·0 mg. of muscle (final dilution 1:10 000–1:100 000). The effect of the reactants present in the extract is negligible at these dilutions. Addition of purified creatine phosphokinase to the crude muscle extract resulted in precise summation of activities. The method will, however, be less accurate in conditions where the acid liberated in the absence of creatine is large compared with that in the assay procedure, i.e. when other enzymes liberating acid are in excess of the creatine phosphokinase.

DISCUSSION

The method described enables creatine phosphokinase activity to be determined in high dilution with great accuracy in about 10 min. The procedure is simple and does not depend upon the use of other enzyme systems, thus making it suitable for inhibitor studies. Provided that the standard conditions are adhered to, the method is specific for creatine phosphokinase. Adenosine triphosphatase activity in the sample to be tested may be accurately estimated by omitting creatine from the reaction system, and can then be subtracted from the total rate to give creatine phosphokinase activity. The main limitation is imposed by the necessity of working at pH 9·0, since at a lower pH the stoicheiometry of hydrogen-ion liberation fails.

It seems likely that the protection afforded by thiols and cyanide against the spontaneous decay in reaction rate is due to maintenance of the thiol groups of the enzyme in the reduced form. However, it is possible that these agents exert their effect by formation of a complex with trace amounts of heavy metals; silver is known to inhibit creatine phosphokinase in very high dilutions

(Martell & Calvin, 1952) and does not form a complex well with ethylenediaminetetra-acetic acid (Solvonuk, McRea & Collier, 1956).

The literature on the relative activating effect of Mn^{2+} and Mg^{2+} ions is not consistent, but Ca^{2+} ion has generally been found to be less effective (Ennor & Morrison, 1958). Part of this disagreement probably arises from the use of glycine as a buffer, since this forms a complex with Mn^{2+} ion more powerfully than with Mg^{2+} ion (Albert, 1950). In the present work Mn^{2+} ion was not only more effective than Mg^{2+} ion, but the rate of the reaction was more nearly constant with both Ca^{2+} and Mn^{2+} ions than with Mg^{2+} ion. The effectiveness of these ions as activators therefore parallels the stability of their complexes with ATP, adenosine diphosphate and creatine phosphate (Smith & Alberty, 1956). The better linearity achieved with Ca^{2+} and Mn^{2+} ions may be related to the severe substrate inhibition of the back-reaction by adenosine diphosphate which occurs with Ca^{2+} ion as the activator (Rosenberg & Ennor, 1955) but not with Mg^{2+} ion (Kuby *et al.* 1954). If this were the case, the rate of the back-reaction would not increase so rapidly as the phosphorylation of creatine proceeds, and the net rate would be more constant.

SUMMARY

1. A titrimetric method for assay of creatine phosphokinase is described.

2. The effects of various protective agents have been investigated and 2-mercaptoethanol and mercaptoacetic acid were found to afford the best protection.

3. The activating effects of various metals have also been studied and Mn^{2+} ion was found to be the most effective.

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REFERENCES

Albert, A. (1950). *Biochem. J.* **46**, xxxix.
Askonas, B. A. (1951). Ph.D. Dissertation: Cambridge University.
Banga, I. (1943). *Stud. Inst. med. Chem. Univ. Szeged*, **3**, 59.
Chappell, J. B. & Perry, S. V. (1954). *Biochem. J.* **57**, 421.
Ennor, A. H. & Morrison, J. F. (1958). *Physiol. Rev.* **38**, 631.
Ennor, A. H. & Rosenberg, H. (1954). *Biochem. J.* **57**, 295.
Fisher, R. A. & Yates, F. (1953). *Statistical Tables for Biological, Agricultural and Medical Research*, pp. 27, 80. New York: Hafner.
Fiske, C. H. & Subbarow, Y. (1929). *J. biol. Chem.* **81**, 629.
Kuby, S. A., Noda, L. & Lardy, H. A. (1954). *J. biol. Chem.* **209**, 191.
Kumler, W. D. & Eiler, J. J. (1943). *J. Amer. chem. Soc.* **65**, 2355.
Martell, A. E. & Calvin, M. (1952). *Chemistry of the Metal Chelate Compounds*. New York: Prentice-Hall.
Meyerhof, O. & Lohmann, K. (1928). *Biochem. Z.* **196**, 43.
Narayanaswami, A. (1952). *Biochem. J.* **52**, 295.
Noda, L., Kuby, S. A. & Lardy, H. A. (1954). *J. biol. Chem.* **210**, 83.
Oliver, I. T. (1955). *Biochem. J.* **61**, 116.
Rosenberg, H. & Ennor, A. H. (1955). *Biochim. biophys. Acta*, **17**, 261.
Smith, R. M. & Alberty, R. A. (1956). *J. Amer. chem. Soc.* **78**, 2376.
Solvonuk, P. F., McRea, S. C. & Collier, H. B. (1956). *Canad. J. Biochem. Physiol.* **34**, 481.

Biochem. J. (1960) **75**, 119

Determination of Riboflavin and its Coenzymes in Tissues

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The determination of riboflavin and its coenzymes in tissues is usually done by the fluorimetric method of Bessey, Lowry & Love (1949). Although this method represents an advance on previous procedures it has some disadvantages, such as the burden of separate determinations of flavine mononucleotide and riboflavin, the high salt concentration after hydrolysis which quenches fluorescence to a considerable extent, and the rather long time needed for a complete determination.

Recently we described methods permitting separation and analysis of flavin mixtures in microgram quantities (Cerletti & Siliprandi, 1955) and on a preparative scale (Cerletti, Montesi & Siliprandi, 1957), and have studied the properties of pure flavin nucleotides (Cerletti & Siliprandi, 1958; Cerletti, 1959).

The data acquired have allowed us to work out an easy and rapid method for the separate determination of riboflavin and its coenzymes in biological material, which will be described here.

Data are given for the influence of salt concentration and of various ions on the fluorescence of flavins and for the variation of fluorescence with the concentration of the fluorescent substance. Illustrative data include also the flavin content of various animal tissues.

MATERIALS AND METHODS

Riboflavin was kindly supplied by Hoffman–La Roche and Co. and riboflavin 5′-pyrophosphate by F. F. Firma, Florence. Flavin mononucleotide was prepared according to Viscontini, Ebnöther & Karrer (1952), flavinadenine dinucleotide according to Cerletti & Siliprandi (1958). Riboflavin 5′-pyrophosphate and flavin mononucleotide were ultimately purified by chromatography on a cation exchanger as described by Cerletti *et al.* (1957).

The qualitative determination of flavins was performed by paper electrophoresis as previously described (Cerletti & Siliprandi, 1955). The quantitative determination of pure flavin solution was obtained by measuring E at 260 and 450 mμ in a Beckman DU spectrophotometer.

Fluorimetric measurements were made in a Klett fluorimeter, lamp filter Corning glass 5543 and photocell filter Corning 3385. Unless otherwise stated, a solution containing 0·079 μg. of fluorescein/ml. was used as standard.

Flavins extracted from tissues were separated on columns, 0·8 cm. × 14 cm., packed to a height of about 7 cm. with 1·01 g. (dry wt.) of Amberlite IRC 50-H, 120–150 mesh. The resin was supported by a small plug of glass wool. After each separation the column was emptied and the cation exchanger was regenerated.

RESULTS

Influence of salt concentration and of ions on the fluorescence of flavins. The fluorescence of flavinadenine dinucleotide, flavin mononucleotide, riboflavin 5′-pyrophosphate and of riboflavin was measured in solutions with increasing concentrations of NaCl and KCl.

Each sample was 1·47 μM with respect to the flavin and 0·01 M with respect to potassium phosphate buffer, pH 6·9. Results are given in Fig. 1.

Hydrolysis of flavinadenine dinucleotide. This compound (7 μM) was hydrolysed in the dark at 100° in N-HCl for 5 min. (*A*) or in 0·1 N-HCl for 15 min. (*B*).

After hydrolysis each sample was neutralized with KOH and the fluorescence compared with that of a sample containing the same concentration of KCl, which had not undergone hydrolysis. The final concentration of flavinadenine dinucleotide was 1·4 μM, that of KCl was 0·2 M in *A* and 0·02 M in *B*.

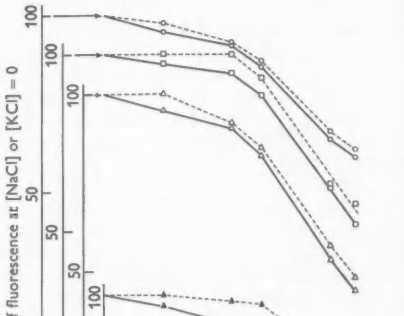

Fig. 1. Effect of increasing concentrations of Na^+ (continuous line) and K^+ (broken line) ions on the fluorescence of flavins. ▲, Flavinadenine dinucleotide; △, flavin mononucleotide; □, riboflavin pyrophosphate; ○, riboflavin. All samples are 0·01 M with respect to phosphate buffer, pH 6·9, and 1·47 μM with respect to the flavin concerned. Molarities of KCl and NaCl and corresponding ionic strengths, including the concentration of buffer, are given on abscissae.

All samples when measured were 0·2 M with respect to potassium phosphate buffer, pH 6·9. The fluorescence before hydrolysis, as compared with that after hydrolysis, was 18·0 % in *A* and 18·5 % in *B*.

Concentration of flavin and fluorescence. Fig. 2 shows the fluorescence of solutions with increasing concentration of flavin and the same concentration (0·03 M) of potassium phosphate buffer, pH 6·8. Within this range of concentration and with the instrument used, a linear relationship of fluorescence to concentration is found.

Determination of flavins in tissues. The tissue, excised immediately after killing the animal, was weighed; to it was added (7 ml./g. wet wt.) ice-cold 0·01 M-KCl–(0·03 M-KH_2PO_4–KH_2PO_4 buffer, pH 6·8) (96 : 4), and it was homogenized at 0° and in the dark with a Potter homogenizer (A.H.T. Co., Philadelphia, U.S.A. no. C-1032). The homogenate was transferred to a tube in a bath at 80° and stirred for 12 min. at this temperature. Evaporation and light were carefully avoided. The homogenate was then rapidly cooled and centrifuged at 18 000 g at 0°. Refrigeration during centrifuging,

though advisable, was not found essential. The supernatant was collected and the precipitate homogenized in the same volume as previously used (7 ml./g. of fresh tissue) of 0·013 M-buffer (4 parts of water and 3 parts of 0·03 M-buffer) and extracted as above. After centrifuging, a third extraction in diluted buffer was carried out on the precipitate as for the second extraction.

The supernatants from the three extractions were mixed and one portion of 4 ml. (A + B) and two of 2 ml. (C and D) were taken.

Portion A + B was adsorbed onto the ion-exchange column and eluted with water at a flow rate of 0·5–1 ml./min. The first 8 ml. of the eluate were collected and divided into 4 ml. fractions in graduated tubes (A and B). Samples C and D were brought to 4 ml. with water.

Samples A and C had 1 ml. of 0·5 N-HCl added and were hydrolysed for 15 min. in a boiling-water bath; they were then cooled and neutralized with 1 ml. of 0·495 N-KOH. The final volume was made to 10 ml. with 0·03 M-K_2HPO_4–KH_2PO_4 buffer, pH 6·8, the final pH being between 4·5 and 6·8. Samples B and D received the same amounts of HCl, KOH and buffer, mixed together before addition.

The distribution of flavins in the samples was as follows: B: flavin mononucleotide + flavinadenine dinucleotide; D: flavin mononucleotide + flavin-adenine dinucleotide + riboflavin; A and C: before hydrolysis as B and D respectively; flavinadenine dinucleotide disappeared after hydrolysis, being quantitatively converted into flavin mononucleotide. Some riboflavin was also formed, but since the fluorescent efficiency of this compound equals, on a molar basis, that of flavin mononucleotide, the fluorimetric determination was not hindered.

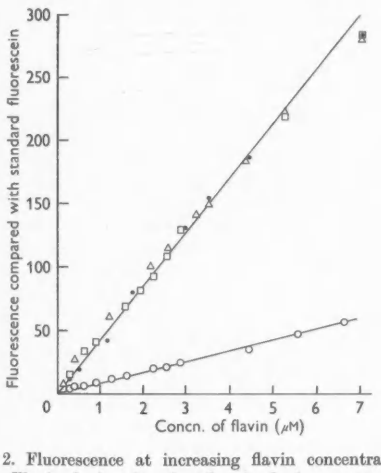

Fig. 2. Fluorescence at increasing flavin concentration. ○, Flavinadenine dinucleotide; △, flavin mononucleotide; ●, riboflavin pyrophosphate; □, riboflavin. All samples are 0·03 M with respect to potassium phosphate buffer, pH 6·8. The fluorescein standard (0·158 μg./ml.) gave a reading of 50.

The fluorescence of each sample was measured: (1) after addition of buffer (F_1); (2) after addition of 1 μg. of riboflavin in 1 ml. of water (F_2); (3) after addition of 0·01 vol. of 10% $Na_2S_2O_4$ in 5% $NaHCO_3$ (F_3). F_2 and F_3 are corrected for dilution (F'_2 and F'_3) and the apparent riboflavin content of each sample is calculated as follows:

$$1\ (\mu\text{g. of riboflavin added}) \times \frac{F_1 - F'_3}{F'_2 - F_1} = F_A, F_B, F_C, F_D \text{ respectively.}$$

Since under our experimental conditions the fluorescence of flavinadenine dinucleotide before hydrolysis is 18% of that after hydrolysis, the real amounts of each flavin in the tissue are calculated as follows:

Riboflavin (μg./g. of tissue wet wt.)
$$= 10{\cdot}5\ (F_D - F_B) = 10{\cdot}5\ (F_C - F_A).$$

Flavinadenine dinucleotide (μg./g. of tissue wet wt.)
$$= \frac{F_A - F_B}{0{\cdot}82} \times 10{\cdot}5 \times \frac{785}{376} = 26{\cdot}73\ (F_A - F_B)$$
$$= \frac{F_C - F_D}{0{\cdot}82} \times 10{\cdot}5 \times \frac{785}{376} = 26{\cdot}73\ (F_C - F_D).$$

Flavin mononucleotide (μg./g. of tissue wet wt.)
$$= \left(F_B - \frac{F_A - F_B}{0{\cdot}82}\right) \times 10{\cdot}5 \times \frac{456}{376}$$
$$= 28{\cdot}26F_B - 15{\cdot}52F_A.$$

When known amounts of flavins were added to the homogenate, total recoveries were obtained.

Determination of peptide-bound flavins. Singer, Kearney & Massey (1957) found that the flavin prosthetic group of succinic dehydrogenase, probably flavinadenine dinucleotide, cannot be resolved from the apoenzyme by heat denaturation or treatment with cold acid, and is determined only after proteolytic digestion. To see if this flavin was determined by our method, the precipitate from the third extraction was regularly resuspended in 21 ml. (per g. of fresh tissue) of a solution 3·3 mM with respect to KCl and 8·9 mM with respect to phosphate buffer, adjusted to pH 7·1 and digested with 1 mg. of trypsin (Merck) and 1 mg. of α-chymotrypsin (Sigma Chemical Co.)/g. of fresh tissue. After 4 hr. at 38° in a thermostatically controlled bath under constant agitation the liquid had completely clarified, or else the undissolved particles were centrifuged off, and flavins were determined as described above.

In other experiments the freshly homogenized tissue was divided into two parts, one of which was

Table 1. *Flavins determined in tissues of normal rats after heat-extraction or proteolytic digestion*

Figures in italic type are calculated as μg. of flavinadenine dinucleotide (FAD) or flavin mononucleotide (FMN)/g. of tissue (wet wt.). Other values are calculated as μg. of riboflavin/g. of tissue (wet wt.). *n*, Number of experiments.

	A. Homogenate extracted in buffered salt solution, as described in the text			B. Proteolytic digest of the precipitate remaining from A after the extractions		C. Homogenate directly digested with trypsin and chymotrypsin without previous extractions		D. Precipitate remaining from C extracted as in A	
Tissue	FAD	FMN	Riboflavin	FAD	FMN + riboflavin	FAD	FMN + riboflavin	FAD	FMN + riboflavin
Liver	*n* = 10 *58·96±4·76*	*n* = 10 *8·42±2·37*	*n* = 10	*n* = 4 *0·54±0·20*	*n* = 4	*n* = 4 *56·89±2·34*	*n* = 3		
	28·24±2·28	6·94±1·96	0·77±0·21	0·26±0·11	0·89±0·30	27·25±1·12	7·8±0·51	—	—
Kidney	*n* = 11 *46·24±2·46*	*n* = 11 *13·46±2·09*	*n* = 11	*n* = 9 *1·65±0·44*	*n* = 9	*n* = 2 *49·27*	*n* = 2	*n* = 1	*n* = 1
	22·15±1·18	11·10±1·73	1·57±0·24	0·79±0·21	0·32±0·14	23·6	11·87	0	0
Heart	*n* = 7 *35·49±0·37*	*n* = 7 *2·01±0·53*	*n* = 7	*n* = 5 *3·38±0·89*	*n* = 5	*n* = 2 *38·41*	*n* = 2	*n* = 1 1·92	
	17·00±0·18	1·66±0·44	0·09±0·02	1·62±0·43	0·39±0·07	18·4	1·26	0·92	0
Spleen	*n* = 8 *9·87±0·89*	*n* = 8 *0·17±0·07*	*n* = 8	*n* = 4 *0·31±0·19*	*n* = 5	*n* = 2 *9·41*	*n* = 2		
	4·73±0·43	0·14±0·06	1·09±0·51	0·15±0·09	0·25±0·09	4·51	1·1	—	—
Brain	*n* = 4 *6·03±0·50*	*n* = 4 *0·86±0·09*	*n* = 4	*n* = 4	*n* = 4				
	2·89±0·24	0·71±0·08	0·21±0·03	0	0	—	—	—	—

treated in the usual way (A) and the other directly digested as described above (B). The precipitate from the third extraction of A was also submitted to proteolytic digestion, and flavins were determined in all samples. Results are shown in Table 1.

Flavin content of animal tissues. The flavin content of some tissues of Wistar albino rats fed on a diet supplemented with vitamins of the B group are shown in Table 1. When brain is assayed, turbid supernatants are often obtained in the first extraction: after separation from the sedimented part they are clarified by addition of 0·1 vol. of 20 % trichloroacetic acid, the new precipitate being added to the rest and treated as usual, and the clear supernatant neutralized with KOH. Values for residual flavin determined on proteolytic digests of the extracted tissues are also reported, and are compared with flavins determined on tissues digested without being extracted.

It is seen that a total extraction of flavins is effected by dilute buffered salt solutions: small amounts of residual flavins are detectable, after proteolytic digestion, only in heart.

DISCUSSION

A complete determination can be carried out within 3–4 hr. from the death of the animal. This is a great advantage over previous methods, which require 1 or 2 days.

As described in a previous paper (Cerletti *et al.* 1957), riboflavin can be eluted from the ion exchanger only by a strong acid. This allows a ready separation from flavin mononucleotide, which is difficult to separate with the benzyl alcohol-extraction method. The recovery of flavinadenine dinucleotide and flavin mononucleotide from the ion-exchange column is quantitative in the volume of eluate collected. This has been ascertained with pure solutions and by adding known amounts of flavins to the tissue extract.

The use of an ion exchanger increases specificity as compared with methods based only on differential fluorimetry, and also makes a direct determination of flavinadenine dinucleotide and flavin mononucleotide possible; this is quicker and more accurate than determinations after elution or measurements of fluorescence on paper after electrophoretic or chromatographic separation, as described by Peel (1958) and Yagi (1957).

Some further disadvantages of these methods may be pointed out also in stages before electrophoresis or chromatography; the extraction with phenol requires great accuracy to avoid a quenching of the fluorescence of flavins, and extraction in trichloroacetic acid causes a small but appreciable breakdown of flavinadenine dinucleotide. Moreover, since recoveries after absorption on Florisil are not quantitative, assumptions must be made to calculate the separated flavins.

The extraction of flavins from tissues with dilute buffered salt solutions gives better results than any other method. This agrees with the data of Yagi (1957), who uses water as a first extractant. However, when water is used in our experimental conditions clear supernatants cannot be obtained with some tissues. With heart, spleen and kidney, lower ionic strengths than those described may be used, the values reported being chosen so as to yield clear supernatants with any material. A slight turbidity does not, however, hinder the exact determination of flavins, calculations being based on an internal standard in each sample. Most flavins are extracted with the first extraction. A residual amount is recovered in the second and third extractions. Further extractions are pointless. As seen from Table 1 small residual amounts of flavins are determined as flavinadenine dinucleotide after proteolytic digestion only in the heart, which is consistent with data on succinic dehydrogenases found in the literature.

As seen from the previous sections, the determination of flavinadenine dinucleotide does not require the use of the ion exchanger and can be done on samples C and D. Sample C can be omitted also when all three flavins are separately determined. It is ordinarily used as a check on the hydrolysis of flavinadenine dinucleotide. In experiments in which known amounts of flavins were added to the tissue extract quantitative and equal recoveries were obtained, calculating alternatively on samples A and B, B and D or C and D, C and A.

The hydrolysis of flavinadenine dinucleotide by heat-treatment in an acid medium greatly shortens the time required for a complete assay and creates more favourable conditions for fluorimetry. Moreover, the fluorescence of flavin mononucleotide, either in pure solutions or dissolved in tissue extracts, is not affected by this treatment; this is due to the equal fluorescent efficiency of flavin mononucleotide and riboflavin, on a molar basis. Exposure to light during hydrolysis should, however, be avoided to prevent the formation of degradation products.

The salt concentration of the samples (19·8 mM with respect to buffer and about 50·6 mM with respect to potassium chloride, I 0·09, and I 0·097 in brain extracts, owing to the addition of trichloroacetic acid) is in a range where only negligible quenching of fluorescence is found. Quenching is, however, found at the concentrations corresponding to the method of Bessey *et al.* (1949) (0·8 M with respect to potassium monohydrogen phosphate and 0·6 M with respect to trichloroacetic acid, I 3).

The quenching of flavin fluorescence by electrolytes has been studied by Theorell & Nygaard (1954) and by Rutter (1958). It has been found to be a function both of ionic strength and species. Our data give evidence for an unfavourable influence of Na^+ ion on fluorescence, especially on the dinucleotide. Szent-Györgyi (1957) noticed that the fluorescence of the dye in the liquid transudate from a muscle stained with acridine orange and suspended in potassium chloride or in sodium chloride is quenched by Na^+ ion, and he attributed this fact to an influence of Na^+ ion on water structures.

As seen from Fig. 2 a linear relationship of fluorescence with flavin concentration is found up to values far above those usually found in samples when measured (not more than 1 μg./ml.). A similar relationship was found by Yagi (1957) for the fluorescence of flavin spots on paper. This fact allows the use of an internal standard for any extract analysed. This is not possible with the Bessey *et al.* (1949) method, where no proportional increase of fluorescence is found at higher flavin concentrations.

When working under uniform conditions standard curves can be drawn with known amounts of flavin. The use of an internal standard is, however, safer and simpler.

SUMMARY

1. The influences of flavin concentration, ionic strength and different ions on the fluorescence of riboflavin, riboflavin 5′-phosphate, riboflavin 5′-pyrophosphate and flavinadenine dinucleotide were studied.

2. A method has been worked out for the extraction and separate determination of these compounds from animal tissues, and the results are compared with those obtained after proteolytic digestion of the tissue.

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REFERENCES

Bessey, O. A., Lowry, O. H. & Love, R. M. (1949). *J. biol. Chem.* **180**, 755.
Cerletti, P. (1959). *Analyt. chim. acta*, **20**, 243.
Cerletti, P., Montesi, G. & Siliprandi, N. (1957). *Arch. Sci. biol., Bologna*, **41**, 554.
Cerletti, P. & Siliprandi, N. (1955). *Biochem. J.* **61**, 324.
Cerletti, P. & Siliprandi, N. (1958). *Arch. Biochem. Biophys.* **76**, 214.
Peel, J. L. (1958). *Biochem. J.* **69**, 403.
Rutter, W. J. (1958). *Acta chem. scand.* **12**, 438.
Singer, T. P., Kearney, E. B. & Massey, V. (1957). *Advanc. Enzymol.* **18**, 65.
Szent-Györgyi, A. (1957). *Bioenergetics*, p. 38. New York: Academic Press Inc.
Theorell, H. & Nygaard, A. P. (1954). *Ark. Kemi*, **7**, 205.
Viscontini, M., Ebnöther, C. & Karrer, P. (1952). *Helv. chim. acta*, **35**, 457.
Yagi, K. (1957). *Bull. Soc. chim. Fr.*, p. 1543.

Biochem. J. (1960) **75**, 124

The Transport of Vitamin A in Rat Serum with Special Reference to the Occurrence of Unidentified Metabolites of Vitamin A in the Rat

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Despite the suspected association of vitamin A alcohol with serum proteins, the mechanism of vitamin A transport in serum is still not fully known. Bennhold (1938) was led to believe that both vitamin A and the carotenoids were associated with the globulins, a view which was later supported by Pett & le Page (1940) after alcoholic fractionation of serum. According to Dzialoszynski, Mystkowski & Stewart (1945), however, vitamin A was said to accompany those proteins of the blood which were precipitated by full or three-quarter saturation with ammonium sulphate. Accordingly it was inferred that vitamin A was most likely attached to albumin.

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The interpretation of results obtained from ammonium sulphate fractionation of chicken, cow and pig plasma is complicated by large losses, particularly of vitamin A ester (Ganguly, Krinsky, Mehl & Deuel, 1952). Nevertheless, it appeared from the observations of Ganguly *et al.* (1952) that 40–60% of the vitamin A alcohol in chicken, cow and pig plasma could be recovered from the proteins precipitating at half-saturation, whereas the vitamin A esters seemed to be associated with the proteins precipitated at lower salt concentrations. After fractionation of chicken and rat plasma by dialysis against distilled water, vitamin A ester was found with a protein fraction which precipitated more readily on dialysis than did the protein carrying vitamin A alcohol.

While our work was in progress, Krinsky, Cornwell & Oncley (1956) reported that during flotation of the lipoproteins of human plasma in the ultracentrifuge the vitamin A alcohol–protein complex sedimented together with the other proteins. From their studies on the transport of vitamin A and of the carotenoids in human plasma Krinsky, Cornwell & Oncley (1958) arrived at the view that since albumin, isolated by two different experimental procedures, did not contain significant amounts of vitamin A alcohol, some protein other than albumin was the carrier of the vitamin A in the blood.

The available evidence would suggest therefore that vitamin A is made soluble in the blood by association with a protein.

With the aid of improved techniques now available for the fractionation of serum proteins by preparative electrophoresis and with the aid of [2-^{14}C]vitamin A, it is hoped in this investigation to identify the vitamin A-carrying serum protein (Garbers, Gillman & Peisach, 1958). Thereafter we shall draw attention to the presence of uncharacterized products of vitamin A in the rat's serum and their possible importance in metabolism.

MATERIALS AND METHODS

[2-^{14}C]*Vitamin A*. This was prepared from [2-^{14}C]vitamin A acid by reduction with lithium aluminium hydride after esterification with diazomethane (Garbers, 1956). The [2-^{14}C]vitamin A alcohol (radioactivity: 1·07 mc/m-mole) was dissolved in purified hexane, stabilized with DL-α-tocopherol (5%) and stored at $-20°$ under N_2 until used for the preparation of doses.

Preparation of doses. Peanut oil was extracted with ethanol, residual ethanol in the oil was removed *in vacuo* and the oil then distilled in a 5 in. rotating disk molecular still (Distillation Products Inc., Rochester, N.Y., U.S.A.). The first third of the distillate was discarded and the residue distilled at 200–220° at 0·03 mm. Hg. α-Tocopherol was added to the oil to the extent of 0·1 mg./100 g. [2-^{14}C]-Vitamin A was dissolved in this oil so that each dose (0·2 ml. of oil) contained 100 i.u. of labelled vitamin A.

Treatment of experimental animals. Male albino rats of the Wistar strain were used in all experiments. Vitamin A-deficient animals were produced by feeding the following diet to weanling animals: casein, 15%; dextrinized corn starch (corn starch cooked in boiling water, dried at a low temperature and ground to a fine powder), 71%; brewer's yeast, 10%; Steenbock's salt mixture no. 40 (NaCl, 9·49%; $MgSO_4,7H_2O$, 10%; $Na_2HPO_4,12H_2O$, 14·59%; K_2HPO_4, 28·28%; $CaHPO_4$, 28·29%; calcium lactate pentahydrate, 6·26%; iron citrate hexahydrate, 2·44%; KI, 0·65%), 4%.

(i) Expts. 1–3 and 5. After 5–6 weeks on the vitamin A-free diet the rats started to lose weight, and fat (5–10%) containing vitamin A (Gillman, Norton, Rivett & Sutton, 1956) was included in the diet. The amount of fat consumed by the rats per day contained the equivalent of 2 i.u. of vitamin A/day. With this amount the rats failed to grow (or grew only slowly), but continued to live. When the amount of vitamin A supplemented was decreased, the rats gradually lost weight and died. Before use the animals were fed with the vitamin A-free diet until signs of deficiency developed, whereupon they received five successive daily doses of 100 i.u. of [2-^{14}C]vitamin A in 0·2 ml. of refined peanut oil by stomach tube. Groups of 5, 15, 6 and 18 rats were used for Expts. 1–3 and 5 respectively.

(ii) Expt. 4. A group of 10 male albino rats (average wt. 34 g.) was weaned on a vitamin A-free diet as above. When these rats started to lose weight (average wt. 157 g.), the diet was supplemented twice weekly with 175 i.u. of [2-^{14}C]vitamin A in peanut oil for 6 weeks (average weight at the end of this period 221 g.). The vitamin A administered allowed marginal storage in the liver and a normal blood-vitamin A concentration (Lewis, Bodansky, Falk & McGuire, 1942).

Collection of blood. The rats were anaesthetized with ether 2–43 days after administration of the last dose and blood was collected from the dorsal aorta. The blood was allowed to clot for 2 hr. and the serum was collected and pooled. Under these experimental conditions the serum contained only vitamin A alcohol with little or no vitamin A esters. The serum for electrophoresis fractionation was dialysed at 2° for 12 hr. against the buffer used for the electrophoresis separation. The vitamin A concentrations of the serum did not decrease during the dialysis.

Vitamin A analyses. Serum proteins were denatured by addition of an equal volume of absolute ethanol. The serum–ethanol mixture was extracted once with an equal volume of light petroleum (b.p. below 40°) followed by two further extractions with half the volume of light petroleum (b.p. below 40°). The extracts were combined and evaporated under N_2 in an Evelyn tube, and the vitamin A in the residue was estimated by the Carr–Price colour reaction with the Evelyn spectrophotometer.

Liver samples were ground with Na_2SO_4 and extracted with ether according to the method of Ames, Risley & Harris (1954). The turbidity which sometimes developed after the addition of the Carr–Price reagent was eliminated by filtering a measured portion of the ether extract through alumina and eluting the vitamin A with hexane–ethanol (92:8, v/v) (Thompson, Ganguly & Kon, 1949).

If the vitamin A concentration in serum or tissue was to be calculated from radioactive assay, the vitamin was

extracted similarly, the solvent evaporated and the residue was subjected to combustion. The $^{14}CO_2$ formed was isolated as $Ba^{14}CO_3$. If required, samples of serum or tissue were also made directly to produce $^{14}CO_2$ by combustion, and $Ba^{14}CO_3$ was isolated.

Vitamin A-breakdown products in the urine. Repeated samples of urine were obtained from vitamin A-deficient rats by making a fistula in the bladder. The neck of the bladder immediately below the entrance of the ureters was isolated by blunt dissection and a small incision made in the ventral wall. A polyethylene tube (no. 1) was passed through the incision into the bladder and tied in position with non-absorbable thread. The cannula was exteriorized and led into a glass saddle affixed to the back of the rat (van Zyl, 1957). After 1–2 days the urine was clear and its flow regular. Four days after the operation the rats were fed by stomach tube 500 i.u. of [2-^{14}C]vitamin A in 0·2 ml. of peanut oil. Through the use of a special metabolism cage (C. F. Garbers & C. Gilbert, unpublished work) urine samples were collected from the saddle and blood samples from the tail at fixed intervals. The blood and urine samples were subjected to combustion separately and the $Ba^{14}CO_3$ was isolated and analysed.

Water and the vitamin A-free diet were supplied without restriction to the experimental animals throughout the experiment.

Combustion of samples and assay of radioactivity. Combustion of samples was according to the method of Evans & Huston (1952), the oxidizing mixture of van Slyke, Plazin & Weisiger (1951) being used. The CO_2 formed during the combustion was isolated as $BaCO_3$ and weighed. The CO_2 was generated from the $BaCO_3$ and absorbed in N-NaOH. From this solution the carbonate was precipitated as $BaCO_3$ on 20 cm.^{2} planchets (approx. 3 mg. of $BaCO_3$/cm.^{2}; Peisach, 1959) and counted with 34% efficiency in a proportional-counter converter (Nuclear Measurements Corp., type P.C.C.-10) screened in 3 in. of steel; gas: Tracerlab proportional gas. The results are expressed as counts/min. and are corrected for self-absorption.

Concentration of lipoproteins from rat serum. This was effected by preparative ultracentrifuging of rat serum at solution densities of 1·063 and 1·20 g./ml. in the Spinco model E analytical ultracentrifuge according to the method of de Lalla & Gofman (1954). The lipoprotein fractions were changed by combustion directly to CO_2 for radioactive assay. The sedimented proteins were dialysed against borate buffer, pH 8·6, to remove most of the KBr before combustion.

Fractionation of rat-serum proteins. The method as described by Porath (1956), in which electrophoresis is carried out on columns from which the proteins are subsequently eluted with buffer, was used. The supporting medium was partially acetylated cellulose powder (Campbell & Stone, 1956, 1957). Two columns were used: column *a* (2 cm. × 32 cm.), washed with borate–phosphate buffer (0·0115 M-$Na_2B_4O_7$–0·0155 M-NaH_2PO_4, pH 8·6, *I* 0·05), was developed for 18 hr. at 18 mA and column *b* (2·2 cm. × 39 cm.), washed with veronal buffer (0·1 M-sodium diethylbarbiturate–0·02 M-diethylbarbituric acid, pH 8·6, *I* 0·05), was developed for 40 hr. at 15 mA. The proteins were eluted at a rate of 10–15 ml./hr. Volume of fractions collected: 2·0–2·1 ml. All these manipulations were carried out at 2°. The buffer used was first kept *in vacuo* to remove dissolved gases and then saturated with N_2.

The protein content of the fractions was determined by measurements of *E* at 280 mμ in a Beckman DU or Unicam SP. 600 spectrophotometer. The homogeneity of the fractions was tested by paper electrophoresis or sedimentation experiments or both.

Concentration of protein solutions for sedimentation experiments. If the protein solutions in the borate–phosphate buffer were too dilute for sedimentation experiments, the fractions were concentrated by evaporation in a vacuum desiccator. The proteins dissolved in the veronal buffer were precipitated with ethanol in the presence of Zn^{2+} ions (Cohn *et al.* 1950) as follows: the protein solution eluted from the column with veronal buffer was mixed with an equal volume of precipitating solution (11·502 g. of $ZnSO_4,7H_2O$, 3 g. of acetic acid and 400 ml. of 95% ethanol, diluted to 1000 ml. with water) at −5°. In trial experiments it was found that the pH, measured with the aid of a glass electrode (instrument standardized against phosphate buffer), of the resulting protein suspension diluted with 4 ml. of NaCl solution (0·02 M) was approx. 6·0 (Cohn *et al.* 1950). After precipitation the supernatant was kept, the precipitate was freeze-dried and dissolved in disodium ethylenediaminetetra-acetate solution [3·308 g. of disodium ethylenediaminetetra-acetate and 77·6 ml. of 0·1 N-NaOH made to 1000 ml.; pH 7·0 (measured with the aid of a glass electrode), *I* 0·05].

Sedimentation. Sedimentation runs were made in the Spinco model E analytical ultracentrifuge at room temperature and at top speed (59 780 rev./min.). After the sedimentation runs, all the mother liquors and protein solutions of the fraction analysed were subjected to combustion and assayed for radioactivity.

Paper electrophoresis. Whatman no. 3 MM paper was suspended horizontally over a Perspex rack in a labyrinth-type of apparatus (van Kampen & Zondag, 1955). Proteins were stained with bromophenol blue (Hardwicke, 1954) and the glycoproteins with the periodic acid–Schiff stain (Björnesjö, 1955).

RESULTS

Zone electrophoresis of rat-serum proteins

Expt. 1. *Electrophoresis fractionation of rat-serum proteins with borate–phosphate buffer (Campbell & Stone, 1956, 1957; Porath, 1956): mobilities of the α_1-glycoprotein and the macroglobulin.* Fig. 1 shows the distribution obtained when rat-serum proteins, which were collected from rats 3 days after administration of the last dose, were fractionated by electrophoresis on a partially acetylated cellulose-powder column. A component migrating faster than the albumin in the electric field can be readily observed. Two consecutive fractions, nos. 7 and 8, were pooled and their homogeneity and nature investigated by paper electrophoresis and by sedimentation runs in the ultracentrifuge. Fig. 2 indicates the sedimentation-boundary diagrams of fractions 7 and 8. Fractions 7–18 were investigated similarly and the results of the analysis (Fig. 1) showed that the albumin peak consisted of at least three components, namely the albumin, the macroglobulin and the third com-

ponent which migrated faster than the albumin under the experimental conditions. This last-named component stained positive for a glycoprotein by the method of Björnesjö (1955) and possessed the sedimentation properties of a globulin. When this component was re-examined by paper electrophoresis in the veronal buffer (pH 8·6, *I* 0·05), it possessed a mobility slightly less than that

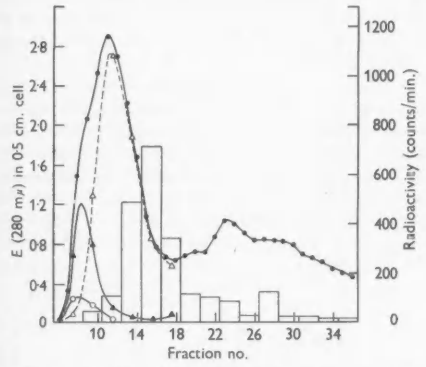

Fig. 1. Zone-electrophoresis fractionation on a cellulose-powder column (2 cm. × 32 cm.) of rat-serum proteins (3 ml.) with the borate–phosphate buffer (pH 8·6, *I* 0·05). A current of 18 mA was applied for 17 hr. ●, Total protein; △, albumin; ▲, α_1-glycoprotein; ○, macroglobulin. Histograms indicate radioactivity. The curve for the α_1-glycoprotein was obtained from the experimentally determined points together with the assumption that at any point (from fractions 6 to 16) the sum of the concentrations of the albumin, α_1-glycoprotein and macroglobulin (determined with the aid of sedimentation experiments) equalled the concentration of the total protein (determined spectroscopically).

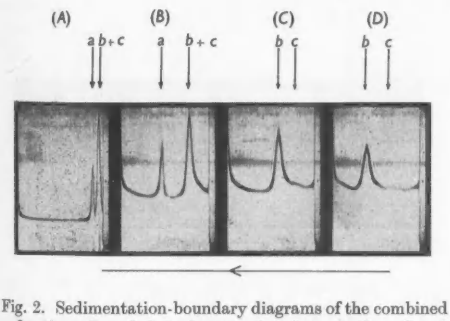

Fig. 2. Sedimentation-boundary diagrams of the combined fractions 7 and 8 in borate–phosphate buffer. Speed 59 780 rev./min. Exposures *A*, *B*, *C* and *D* were taken 8, 32, 56 and 80 min. respectively after reaching top speed. *a*, Macroglobulin; *b*, glycoprotein; *c*, albumin.

Table 1. *Correlation between the vitamin A concentrations of rat-serum as calculated from Carr–Price measurements and radioactive assay*

Values for the vitamin contents of serum are the averages of duplicate analyses unless indicated otherwise.

Expt.	No. of rats	No. of days between feeding of last dose and collection of serum	Vitamin A (i.u./100 ml. of serum) calc. from Carr–Price measurements	Vitamin A (i.u./100 ml. of serum) calc. from Radioactive assay on fat-soluble components (*a*)	Vitamin A (i.u./100 ml. of serum) calc. from Radioactive assay on serum (*b*)	Percentage of activity not extractable	*b*/*a*	Counts/min. on α_1-globulin (*c*)	Counts/min. on globulins (*d*)	*d*/*c*
1 (Fig. 1)	5	3	111	112	121	7·5	1·08	2247	2247	1
2 (Fig. 3)	15	2	101	—	131	23*	1·30*	1446	1977	1·37
3 (Fig. 4)	6	4	53†	45·6†	75	39·2	1·64	970	1530	1·58
4 (Fig. 6)	7	7	Lost	135	189·8	29	1·41	2440	3640	1·49
4	3	43	74†	66·7	86·2	22·6	—	—	—	—
5	13	2	101	—	134	24·6*	—	—	—	—
5	5	10	73	—	100·1	27·1*	—	—	—	—

* Calculated from radioactive assay and Carr–Price measurements on serum.
† Results from single analysis on serum.

of the albumin and is therefore most likely an α_1-glycoprotein (Garbers & Joubert, 1958). Analyses of the fractions revealed the radioactivity to be associated with the α_1-globulin. The radioactivity is not associated with the albumin, since one would then expect the radioactivity to be proportionally distributed over the albumin peak (Fig. 1).

The vitamin A content of the serum in Expt. 1 calculated from Carr–Price measurements and radioactive assay on whole serum and the fat-soluble components from serum agree well (Table 1).

Expt. 2. *Electrophoresis fractionation of rat-serum proteins with borate–phosphate buffer: isolation of the lipoproteins.* Fig. 3 shows the result when the serum-protein fractions eluted from the column after electrophoresis were analysed for radioactivity. The radioactivity was identified not only on the α_1-globulin but also on other globulins. From the latter approx. 27 % of the total radioactivity was eluted (Table 1). It is possible that some of the radioactivity was carried on the β-globulin (Fig. 3).

The lipoproteins from the same serum were also isolated by flotation in the ultracentrifuge according to the method of de Lalla & Gofman (1954). The radioactivity was associated neither with the low- nor with the high-density lipoproteins, but with the proteins which sediment during flotation of the lipoproteins in the ultracentrifuge. The results are summarized in Table 2.

The vitamin A content of the serum obtained by radioactive assay exceeded the vitamin A level as determined by Carr–Price colour reaction by 29·7 % (Table 1).

Expt. 3. *Electrophoresis fractionation of rat-serum proteins with veronal buffer: mobility of the macroglobulin in this buffer.* Fig. 4 shows the distribution obtained when rat serum, obtained from the rats 4 days after administration of the last dose, was fractionated by zone electrophoresis in veronal buffer.

In Expt. 1 it was established that when the borate–phosphate buffer is used for the electrophoretic fractionation of rat-serum proteins, two components (macroglobulin and α_1-glycoprotein) migrated faster than the albumin. The ultracentrifugal analysis of the pooled fractions 15 and 16 of this experiment reveals that when the non-complexing veronal buffer is employed for the

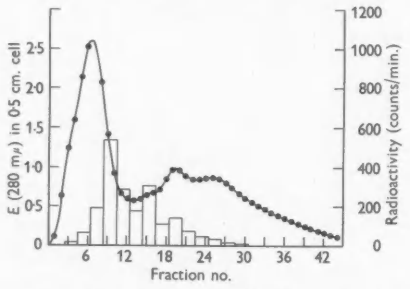

Fig. 3. Zone-electrophoresis fractionation of rat-serum proteins (3 ml.). Conditions were identical with those of Fig. 1. A current of 18 mA was applied for 18 hr. ●, Total protein. Histograms indicate radioactivity.

Table 2. *Isolation of lipoprotein fractions*

Description of fractionation	Radioactivity (%) in: Lipoprotein fraction	Radioactivity (%) in: Sedimented proteins
Ultracentrifugal flotation fractionation at solution density 1·063	1·1	98·1
Ultracentrifugal flotation fractionation at solution density 1·20	3·9	94·2

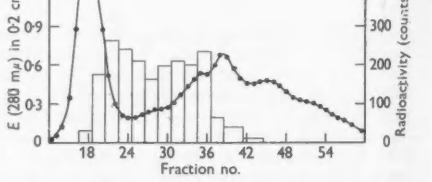

Fig. 4. Zone-electrophoresis fractionation of rat-serum proteins (3·5 ml.) on a cellulose-powder column (2·2 cm. × 36 cm.) with veronal buffer (pH 8·6, *I* 0·05). A current of 15 mA was applied for 36 hr. ●, Total protein. Histograms indicate radioactivity.

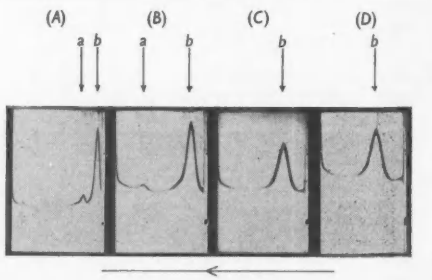

Fig. 5. Sedimentation-boundary diagrams of the combined fractions 15 and 16 in ethylenediaminetetra-acetic acid solution. Speed 59 780 rev./min. Exposures *A*, *B*, *C* and *D* were taken 8, 32, 56 and 80 min. respectively after reaching top speed. *a*, Macroglobulin; *b*, albumin.

electrophoresis of rat serum, only the macroglobulin is found to migrate faster than the albumin (Fig. 5).

Analysis of the protein fractions revealed that approx. 63·4% of the radioactivity recovered in these fractions was associated with the α_1-globulin and the remainder with other globulins.

Here again the vitamin A concentrations differed when determined by Carr–Price colour reaction and by radioactive assay on the whole serum. It was found that 39·2% of the radioactivity was not extractable from serum by the conventional methods employed during the Carr–Price estimation (Table 1).

Expt. 4. Electrophoresis fractionation of rat-serum proteins with veronal buffer: mobility of the α_1-glycoprotein in this buffer. The vitamin A administered to the rats in Expts. 1–3 and 5 was not sufficient to allow storage. The association of vitamin A with the serum proteins in rats having reserves of vitamin A in the liver was investigated. Under the experimental conditions described, the rat livers contained an average of 31·2 i.u. of vitamin A/g. of liver (range 21–44 i.u./g.) 1 week after administration of the last dose. The analysis of the serum is reported in Table 1.

A procedure similar to that described in Expt. 3 was followed for the fractionation of a sample of pooled serum obtained from a group of seven rats 1 week after administration of the last dose. When the protein fractions eluted from the column after electrophoresis were analysed, 67% of the radioactivity recovered was associated with the α_1-globulin and the remaining 33% with the other globulins (Fig. 6).

The position of the α_1-glycoprotein was determined by spotting on paper portions of the fractions, obtained after electrophoresis and staining for glycoproteins, followed by elution and subsequent quantitative estimation of the dye as recommended by Björnesjö (1955). As is expected from an α_1-globulin component, the mobility of the glycoprotein is smaller than that of the albumin (Fig. 6). The presence of globulin components could also be demonstrated by paper-electrophoresis techniques in fractions 14–20 of the albumin peak. In the veronal buffer the macroglobulin still migrates faster than the albumin (Expt. 3), whereas in the borate–phosphate buffer both the α_1-glycoprotein and the macroglobulin migrate faster than the albumin during electrophoresis (Expt. 1).

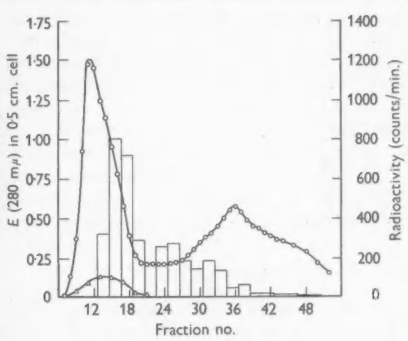

Fig. 6. Zone-electrophoresis fractionation of rat-serum proteins (4 ml.) on a cellulose-powder column (2·2 cm. × 36 cm.) with the veronal buffer (pH 8·6, I 0·05). A current of 15 mA was applied for 40 hr. ○, Total protein; △, glycoprotein. Histograms indicate radioactivity.

It has been observed before that the mobilities of certain components in sera change in buffers containing borate (Consden & Powell, 1955; Goldwasser & Mathews, 1955; Aronsson & Grönwall, 1957; Cooper, 1958). This increased mobility of the α_1-glycoprotein is most likely due to the action of the borate ion with the glycol groupings, leading to increased charges on the glycoproteins. As the position of the peak of radioactivity on the α_1-globulin is unaltered when either the borate–phosphate buffer or the veronal buffer is employed, the radioactivity is probably also not associated with the α_1-glycoprotein.

Influence of time on the amount of radioactivity not extractable from serum

The results obtained in Expts. 1–3, where serum samples were collected 3, 2 and 4 days respectively after administration of the last dose of labelled vitamin A, indicated that the radioactivity could not always be completely extracted by the conventional methods used for the extraction of vitamin A from serum. A similar investigation (Expt. 5) verified these results, and a difference of 24·6 and 27·1% still existed between the vitamin A concentration of the serum determined by Carr–Price colour reaction and that calculated from radioactive assay 2 and 10 days after administration of the last dose (Table 1).

Similarly, in Expt. 4, where the experimental animals had reserves of labelled vitamin A in the liver, a discrepancy was found between the radioactivity in whole serum and the radioactivity in the fat-soluble components extracted from serum 1 week after administration of the last dose. To assess whether this discrepancy is persistent in serum, three rats from this group were maintained on a vitamin A-free diet for a period of 43 days after administration of the last dose. During this period the rats continued to grow (average increase in weight: 22 g.), and at the end of this period 29·2% more radioactivity was found in whole serum than in the fat-soluble components extracted from the serum (Table 1).

Comparison of the concentrations of vitamin A in rat serum as calculated from radioactive assay and from Carr–Price measurements on serum

In the paragraph 'Treatment of experimental animals' conditions are described that enabled us to create a physiological condition which allowed the animal to survive without having any vitamin A in the blood or tissues as judged by the Carr–Price colour reaction. Administration of 500 i.u. of labelled vitamin A to these animals resulted in the appearance of vitamin A in the blood. This vitamin A was all labelled, since the vitamin A content of the fat-soluble components extracted from the serum was of the same order whether determined by radioactive assay or Carr–Price colour reaction. However, on occasions not all the radioactivity was confined to the fat-soluble extracts. In other words, part of the radioactivity derived from the labelled vitamin A was present as a component (or components) not extractable from serum by conventional methods. Furthermore, this second labelled component(s) could not be separated from the serum by dialysis.

The normal occurrence of this second labelled component(s) in the serum was suspected from the results of Expts. 4 and 5. Despite the varying conditions under which these experiments were conducted, this second labelled component(s) was still present in quantities varying from 23 to 29% of the total radioactivity in serum (Table 1).

The large amount of radioactivity (39·2%) which could not be extracted from the serum in Expt. 3 was found in rats greatly depleted of vitamin A before the labelled vitamin A was fed. In this connexion it should be mentioned that during the period of depletion and before radioactive vitamin A was fed one rat developed panophthalmia.

If the results presented in Table 1 are considered with the data obtained from the electrophoresis fractionation of the serum proteins, it is evident that: (i) one peak of radioactivity on the α_1-globulin is to be found when the vitamin A concentrations of the serum calculated from the radioactive assay and from the Carr–Price estimation correlate well (Fig. 1); (ii) a discrepancy between the vitamin A concentration calculated from total radioactivity and from the Carr–Price measurements on whole serum was found with two peaks of radioactivity, namely one on the α_1-globulin and the second on other globulins.

No proof can be offered that part or all of the radioactivity eluted from the column was indeed present as vitamin A. However, the correlations presented in Table 1 more than suggest that the fat-soluble components extracted from the ethanol-denatured serum contain vitamin A alcohol as the only radioactive component on the α_1-globulin. The radioactivity not extractable from the ethanol-denatured serum is associated mostly with the α_2-globulin.

Breakdown products of vitamin A in the urine

Labelled water-soluble products (11·7% of the dose) derived from [2-^{14}C]vitamin A are excreted in the urine of rats within 24 hr. after the intraperitoneal injection of 9200 i.u. of [2-^{14}C]vitamin A to depleted rats (Wolf, Kahn & Johnson, 1957).

Under our experimental conditions the excretion of radioactivity in the urine reached a maximum during the period 4–10 hr. after the oral administration of 500 i.u. of [2-^{14}C]vitamin A (Fig. 7). In the two experiments conducted, the radioactivity excreted in the urine during the 48 hr. after oral administration was 6·8 and 11·5% of the administered dose. The bulk of this radioactivity, 69·4 and 82·4% respectively, was excreted during the first 24 hr. period.

The radioactivity in the blood reached a maximum after approx. 4 hr., and at the end of 48 hr. the livers of the animals contained radioactivity corresponding to 20·6 and 19·8% respectively of the administered dose.

Fig. 7. Appearance of radioactivity in the blood and urine of vitamin A-deficient rats after administration of 500 i.u. of [2-^{14}C]vitamin A. Rat 1: ●, radioactivity in whole-blood samples; ○, radioactivity in urine samples. Rat 2: △, radioactivity in whole-blood samples; ▲, radioactivity in urine samples.

DISCUSSION

Our investigations in the rat disclose that vitamin A alcohol is transported by a globulin component of the serum protein. In this respect the transportation of vitamin A in the rat is similar to that described for man by Krinsky *et al.* (1958). In the rat the globulin component could be still further characterized. The vitamin A alcohol appears to

accompany the α_1-globulin but probably not the α_1-glycoprotein.

The finding that vitamin A alcohol is not associated with the lipoproteins clearly dissociates the transporting mechanism of vitamin A alcohol from that of the vitamin A esters, which are transported in rat serum, as in man, by the low-density lipoproteins (Garbers, 1958).

The amount of vitamin A in rat serum, as determined by Carr–Price colour reaction, cannot always account for all the radioactivity in the serum after the oral administration of labelled vitamin A to depleted rats. Another component(s) derived from vitamin A therefore is present in the blood. Unfortunately, in this investigation no evidence can be presented as yet whether there is one or more such component. The results to date indicate that in the serum of rats with little or no reserves of vitamin A the concentration of this second component(s) is equivalent (in terms of radioactivity) to approx. 20–30 i.u. of vitamin A/100 ml. of serum. The transport of the second component(s) derived from vitamin A seems to be different from that of vitamin A alcohol.

The evidence for and against the presence in serum of an intermediary product of vitamin A not detectable by the methods usually employed for the chemical estimation of vitamin A has been reviewed (Moore, 1957). Experiments suggest that on feeding vitamin A to depleted rats the vitamin A absorbed is first transformed into an intermediary product which in turn is utilized for promoting growth and for preventing xerophthalmia (Moore, 1957). Once this intermediary product reaches a particular concentration, vitamin A alcohol may be expected in the blood and ultimately in the liver and kidneys.

A situation can be visualized where rats are depleted of their stores of vitamin A and eventually also of the vitamin A in the blood. In such circumstances the intermediary product nevertheless may still be present in the blood, thus accounting for survival of vitamin A-deficient rats despite the failure to demonstrate vitamin A in the blood by conventional methods. On feeding labelled vitamin A to these rats, the labelled vitamin A again appears in the blood and depots. However, in short-term experiments the intermediary product may still not be labelled. In such circumstances there may be adequate amounts of the intermediate, i.e. the more immediately utilizable form of vitamin A. Accordingly the vitamin A administered to the depleted rat is not required for metabolism and therefore the vitamin A concentration rises in the blood and also later in the depots. On the other hand, depending on the state of depletion of the animal and the amount of labelled vitamin A fed, a situation could arise where there is so little of the vitamin A intermediate present in the blood that the feeding of labelled vitamin A would lead rapidly to the formation and appearance in the blood of the labelled vitamin A intermediate at the same time as the labelled vitamin A alcohol accumulates in the blood. The finding that this second labelled component(s) occurs in varying quantity in rat serum does not necessarily exclude it (them) from being a normal product(s) of vitamin A metabolism.

le Gallic (1947) provided suggestive experimental evidence for the presence in the blood of albino rats of a component having vitamin A bioactivity, but which was not detectable by the Carr–Price colour reaction. Since le Gallic's experiments and the investigations of others of the 'lard factor', the so-called 'hidden forms' of vitamin A have received renewed attention. It has now been shown that herring eggs also possess more bioactivity than can be accounted for by the Carr–Price colour reaction (Fisher *et al.* 1956). Subsequently, Plack, Thompson & Kon (1958) and Plack, Kon & Thompson (1959) identified vitamin A aldehyde as a constituent of herring eggs. Evidence which might indicate the presence of retinene in the rat's liver was also presented (Scharpenseel & Wolf, 1958).

In our experiments, this second labelled component(s) in the rat serum may be merely a breakdown product(s) of vitamin A and not an active intermediate product(s) of vitamin A. It is known that compounds similar in structure to vitamin A are absorbed, transported in the blood and stored in the liver (Ames, Swanson & Harris, 1955).

The rat normally does not excrete any vitamin A in the urine. Furthermore, the labelled products in the urine after feeding [2-^{14}C]vitamin A are water-soluble (Wolf *et al.* 1957) and may be either the active intermediate(s) or merely a breakdown product(s) thereof or of vitamin A. If the labelled products in the urine are breakdown products of vitamin A, they are most likely derived from the circulating blood. However, there is a possibility that the labelled products are elaborated and excreted by the kidneys or, less likely, by the urinary passages. However, if the component(s) in the urine is the same as that in the serum, it follows from the results obtained that the kidneys have the ability to concentrate this component(s), especially as the average excretion of radioactivity/unit volume in the urine is greater than in the blood, where the radioactivity is due to [2-^{14}C]-vitamin A and the other component(s). As the synthetic vitamin A was labelled at $C_{(2)}$, the second labelled component(s) in the serum is to be expected to be relatively large or to be covalently linked to the proteins, otherwise it (they) should separate during dialysis.

Whether this second labelled component(s) in rat serum belongs to the hidden forms of vitamin A or whether it (they) is merely a decomposition product is now being investigated in this Laboratory.

SUMMARY

1. With the aid of [2-^{14}C]vitamin A it was established that vitamin A alcohol is transported in rat serum in association with the α_1-globulin. The vitamin A alcohol is not associated with the lipoproteins.

2. When a borate–phosphate buffer is employed for the electrophoretic fractionation of rat-serum proteins, the mobility of the α_1-glycoprotein is greater than that of the albumin.

3. By employing veronal buffer and borate–phosphate buffer for the electrophoretic fractionation of rat-serum proteins, it could be concluded that vitamin A alcohol is probably also not associated with the α_1-glycoprotein.

4. A labelled component(s) derived from [2-^{14}C]-vitamin A and associated with the α_2-globulin can occur in serum and most likely is a normal constituent of serum, occurring in a concentration equivalent to 20–30 i.u. of vitamin A. It has not been possible as yet to establish whether these derivatives of vitamin A are active intermediates or degradation products.

5. On giving [2-^{14}C]vitamin A orally to vitamin A-depleted rats, 7–12% of the radioactivity is excreted in the urine within 48 hr. after administration.

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REFERENCES

Ames, S. R., Risley, H. A. & Harris, P. L. (1954). *Analyt. Chem.* **26**, 1378.

Ames, S. R., Swanson, W. J. & Harris, P. L. (1955). *J. Amer. chem. Soc.* **77**, 4136.

Aronsson, T. & Grönwall, A. (1957). *Scand. J. clin. Lab. Invest.* **9**, 338.

Bennhold, H. (1938). *Die Eiweisskörper des Blutplasmas.* Dresden and Leipzig: Theodor Steinkopf.

Björnesjö, K. B. (1955). *Scand. J. clin. Lab. Invest.* **7**, 153.

Campbell, P. N. & Stone, N. E. (1956). *Biochem. J.* **62**, 9P.

Campbell, P. N. & Stone, N. E. (1957). *Biochem. J.* **66**, 19.

Cohn, E. J., Gurd, F. R. N., Surgenor, D. M., Barnes, B. A., Brown, R. K., Derouaux, G., Gillespie, J. M., Kahnt, F. W., Lever, W. F., Liu, C. H., Mittelman, D., Mouton, R. F., Schmid, K. & Uroma, E. (1950). *J. Amer. chem. Soc.* **72**, 465.

Consden, R. & Powell, M. N. (1955). *J. clin. Path.* **8**, 150.

Cooper, D. R. (1958). *Nature, Lond.*, **181**, 713.

de Lalla, O. & Gofman, J. W. (1954). *Meth. biochem. Anal.* **1**, 459.

Działoszynski, L. M., Mystkowski, E. M. & Stewart, C. P. (1945). *Biochem. J.* **39**, 63.

Evans, E. A. & Huston, J. L. (1952). *Analyt. Chem.* **24**, 1482.

Fisher, L. R., Harrison, G. F., Kon, S. K., Plack, P. A., Thompson, S. Y. & Todd, P. E. E. (1956). *Proc. Nutr. Soc.* **15**, xi.

Ganguly, J., Krinsky, N. I., Mehl, J. W. & Deuel, H. J. (1952). *Arch. Biochem. Biophys.* **38**, 275.

Garbers, C. F. (1956). *J. chem. Soc.* p. 3234.

Garbers, C. F. (1958). *Nature, Lond.*, **182**, 1018.

Garbers, C. F., Gillman, J. & Peisach, M. (1958). *S. Afr. J. med. Sci.* **23**, 34.

Garbers, C. F. & Joubert, F. J. (1958). *Nature, Lond.*, **182**, 530.

Gillman, J., Norton, K. B., Rivett, D. E. A. & Sutton, D. A. (1956). *Biochem. J.* **63**, 458.

Goldwasser, E. & Mathews, M. B. (1955). *J. Amer. chem. Soc.* **77**, 3135.

Hardwicke, J. (1954). *Biochem. J.* **57**, 166.

Krinsky, N. I., Cornwell, D. G. & Oncley, J. L. (1956). *Fed. Proc.* **15**, 113.

Krinsky, N. I., Cornwell, D. G. & Oncley, J. L. (1958). *Arch. Biochem. Biophys.* **73**, 233.

le Gallic, P. (1947). *C.R. Soc. Biol., Paris*, **141**, 1214.

Lewis, J. M., Bodansky, O., Falk, K. G. & McGuire, G. (1942). *J. Nutr.* **23**, 351.

Moore, T. (1957). *Vitamin A*, p. 285. Amsterdam: Elsevier Publishing Co.

Peisach, M. (1959). *J. S. Afr. chem. Inst.* **12**, 57.

Pett, L. B. & le Page, G. A. (1940). *J. biol. Chem.* **132**, 585.

Plack, P. A., Kon, S. K. & Thompson, S. Y. (1959). *Biochem. J.* **71**, 467.

Plack, P. A., Thompson, S. Y. & Kon, S. K. (1958). *Biochem. J.* **68**, 2P.

Porath, J. (1956). *Biochim. biophys. Acta*, **22**, 151.

Scharpenseel, H. W. & Wolf, G. (1958). *Fed. Proc.* **17**, 491.

Thompson, S. Y., Ganguly, J. & Kon, S. K. (1949). *Brit. J. Nutr.* **3**, 50.

van Kampen, E. J. & Zondag, H. A. (1955). *Chem. Weekbl.* **51**, 535.

van Slyke, D. D., Plazin, J. & Weisiger, J. R. (1951). *J. biol. Chem.* **191**, 299.

van Zyl, A. (1957). *J. Endocrin.* **16**, 213.

Wolf, G., Kahn, S. G. & Johnson, B. C. (1957). *J. Amer. chem. Soc.* **79**, 1208.

The Physicochemical Requirements for the Action of *Penicillium notatum* Phospholipase B on Unimolecular Films of Lecithin

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Previous work has shown that emulsions of lecithin are attacked by the phospholipase B of *Penicillium notatum* only when they are activated by certain other lipids such as monophosphoinositide or cardiolipin (Dawson, 1958*a*). It was found that such activators do not operate as typical coenzymes but rather as substances which produce distinct physical changes on the surface of the lecithin particles (Dawson, 1958*b*). A study of the system by electrophoresis has indicated that the phospholipase activity can be correlated with the presence on the lecithin particles of a net negative surface (Bangham & Dawson, 1959*a*). The enzyme activity occurred only when the net negative potential of the lipid particles had reached a certain critical value. This could be obtained not only by the introduction into the lecithin of naturally occurring lipids such as monophosphoinositide but also by the use of synthetic anionic amphipathic molecules (substances having a hydrophobic portion and a polar group), e.g. dicetylphosphoric acid, hexadecyl sulphate.

The enzyme system has also been studied by using surface films of [^{32}P]lecithin floating on a buffer and observing the loss of radioactive counts coming from the surface when enzyme was introduced into the buffer (Dawson & Bangham, 1959). Here again, enzyme activity occurred only when the surface film of lecithin contained a certain minimum proportion of anionic amphipathic molecules. However, in these experiments the lecithin was added in much greater quantities than was necessary to form a unimolecular film, and consequently the excess existed in the form of 'rafts' or liquid crystals presumably separated by a monolayer at its collapse pressure (Adam, 1941).

In the present experiments the enzyme system has been studied by using unimolecular films of [^{32}P]lecithin of high specific activity spread on a Langmuir-type trough with arrangements for continuously recording not only the loss of surface counts (enzyme activity) but also the pressure of the film and the surface potential. With these techniques it has been shown that at low-surface pressures the enzyme can hydrolyse a pure lecithin surface which possesses no apparent surface charge. On increasing the pressure this activity first increases and then at a pressure of about 30 dynes/cm. is sharply abolished. With high-pressure films it can be obtained only if negative groupings are introduced into the surface, e.g. by the addition of dicetylphosphoric acid.

EXPERIMENTAL

Preparation of [^{32}P]*lecithin.* The biosynthetic preparation of [^{32}P]lecithin with *Saccharomyces cerevisiae* previously described (Dawson & Bangham, 1959) has been modified to simplify the procedure for the purification of the extracted yeast lecithin. The yeast was grown in 2 l. of the same nutrient medium with 5 mc of $^{32}PO_4^{3-}$ added. The yeast was harvested at the point when no inorganic P, as measured by the Fiske & Subbarow (1925) method, was left in the medium; Fig. 1 shows that this gives a maximum yield of yeast phospholipid. The extraction of the lipids from the yeast was as described in the previous method (Dawson & Bangham, 1959). The dehydrated lipids were thoroughly extracted with 100 ml. of diethyl ether, and after insoluble matter had been removed by filtration the filtrate was evaporated to dryness under reduced pressure

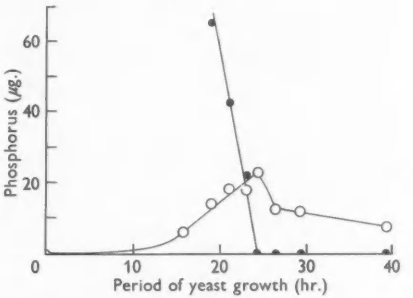

Fig. 1. Accumulation of phospholipid in bakers' yeast during growth in a medium containing limited inorganic phosphate. The phospholipid was extracted from the yeast cells with $CHCl_3$–methanol and purified by the procedure of Folch, Lees & Sloane-Stanley (1957). ○, Yeast phospholipid P in 40 ml. of medium; ●, inorganic P in 2 ml. of medium.

and the lipid residue extracted with 8 ml. of $CHCl_3$–methanol (1:1, v/v). The extract, together with any insoluble matter, was transferred to an activated alumina column (15 cm. long × 0·8 cm. diam.; Hopkins and Williams Ltd.). Solvent ($CHCl_3$–methanol; 1:1, v/v) was passed down the column; the lecithin emerged almost immediately and was detected by monitoring the effluent with a Geiger–Müller counter. Kephalins and insoluble matter remained on the column. The lecithin solution was evaporated to dryness *in vacuo* and chromatographed on a silicic acid column (10 cm. long × 0·8 cm. diam.; Mallinckrodt Ltd.) with $CHCl_3$–methanol (68:32, v/v). Fractions of 5 ml. were collected with an automatic fraction collector; the neutral lipids appeared almost immediately, and the lecithin, now free from lysolecithin, emerged in (approximately) tubes 18–33 with some tailing afterwards. Its position was easily located by monitoring the ^{32}P in the fractions. The resulting [^{32}P]lecithin was stored at −15° in $CHCl_3$. Analysis by a new hydrolytic method (Dawson, 1960) showed that the only phospholipid present was lecithin; choline plasmalogen could not be detected.

For use, a sample of the solution of [^{32}P]lecithin in $CHCl_3$ was evaporated to dryness *in vacuo* and redissolved in the appropriate volume of light petroleum (b.p. 60–80°) to give a solution containing about 1·2 μmoles/ml. Dicetylphosphoric acid (Albright and Wilson Ltd.) was dissolved in the minimum amount of $CHCl_3$ and diluted with light petroleum before mixing it with the lecithin solution.

Preparation of enzyme. The enzyme was extracted from the *P. notatum* and purified with ammonium sulphate according to a previously described method (Dawson, 1958*a*). The final dialysis was against distilled water at 4° for 3 days. The purified enzyme was more effective in attacking negatively charged monolayers of lecithin than the original extract; evidence was obtained during the investigation that this may be due to the inhibitory effect of positively charged proteins in the original extract.

Apparatus for continuously recording the enzymic hydrolysis, surface pressure and potential of a unimolecular film of lecithin. The basis of the apparatus (Fig. 2) was a trough milled from a block of polytetrafluoroethylene (Fluon, Imperial Chemical Industries Ltd.) of 75 ml. capacity, 20 cm. long, 4·5 cm. wide and 0·83 cm. deep. This was filled to the brim with water adjusted to the pH optimum of the enzyme (3·3) with HCl. The surface was cleaned with a waxed glass sweep. The solution was stirred from below by using a reciprocating magnet moving a glass-sheathed mumetal-wire stirrer. Sufficient [^{32}P]lecithin (approx. 10^{16} molecules) dissolved in light petroleum (b.p. 60–80°) to form a monolayer was added to the surface by means of an Agla microsyringe.

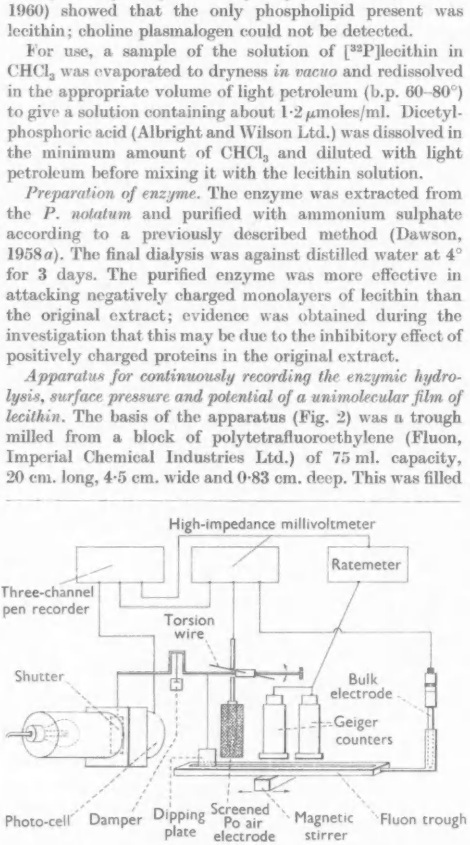

Fig. 2. Diagram of the apparatus for the simultaneous measurement of the radioactivity, surface pressure and potential of a monolayer of [^{32}P]lecithin.

The pressure of the surface was adjusted by moving a waxed glass sweep along the trough and was recorded by means of a Wilhelmy (1863) hydrophilic dipping plate (microscope coverslip fused to a platinum wire); this was suspended from an arm of a torsion balance. An extension of the arm operated a shutter in front of a photoelectric cell and the current arising therefrom was recorded continuously by a pen recorder. The torsion balance was calibrated in dynes/cm. of surface pressure by means of a hanging pan and weights. Care was taken to ensure that the contact angle of the liquid on the plate approached zero by careful washing of the dipping plate with ethanolic KOH and concentrated HNO_3 followed by multiple rinses with dilute HCl. The plate was allowed to drain before the torsion balance was calibrated in dynes/cm. or any surface-pressure measurements were made.

The radioactivity of the surface film was measured by two mica end-window β-counter tubes (Mullard MX 123) in parallel and adjusted so that their windows were at a distance of 0·5 cm. from the film. The mica windows were protected from accidental contamination with ^{32}P by a thin polythene sheet. The pulses were integrated (over 20 sec.) in an Ecko ratemeter and the output voltage was fed into a pen recorder.

The surface potential Δv (i.e. the change observed in the potential which exists between an electrode in air and the aqueous phase on spreading a monolayer) was measured with a 12 mc ^{210}Po air-ionizing electrode (Ministry of Supply, The Radiochemical Centre) held 0·5 cm. from the film. The electrode was screened with an earthed cylinder of copper gauze: the aqueous electrode was a standard calomel electrode seated in a polythene extension to the Fluon trough, a measure designed to prevent contamination of the aqueous phase in the trough with KCl from the electrode. The potential from the polonium electrode was fed by low-microphony cable for amplification by a standard Pye Universal pH/millivoltmeter which had an input impedance of 500 MΩ. The output from the millivoltmeter, the ratemeter and the surface-pressure photoelectric cell were plotted continuously with a three-channel Honeywell–Brown DC pen recorder.

The trough, dipping plate, ^{210}Po air electrode and β-counter tubes were contained in a copper-lined incubator so that observations could be made at temperatures above those in the laboratory. It was observed that temperature fluctuation markedly affected the stability of the surface potential, and it was found better to eliminate the thermostatic control on the incubator and feed its heater with a constant low voltage supplied by a variable transformer.

Paper chromatography of the ^{32}P-containing reaction products. A major part of the aqueous phase (50 ml.) under the unimolecular film was withdrawn by suction and cooled in ice. To the solution was added, as carriers, 88 μg. of glycerylphosphorylcholine P (Dawson, 1956) and 52 μg. of purified ovolecithin P. After vigorous shaking a sample (10 ml.) was taken for assay of the total radioactivity. The

remaining 40 ml. was passed down a column of De-Acidite E (15 cm. long × 0·8 cm. diam.) to remove the HCl which had been used to adjust the solution to pH 3·3 (the enzyme optimum). The column had been previously treated with aq. 5 N-NH_3 soln. and was well washed with water. The weakly basic arylamine grouping removed the HCl but not the glycerylphosphorylcholine. The effluent plus 10 ml. of washings was evaporated to dryness in a rotary drier *in vacuo* below 50°. The residue was dissolved in 0·6 ml. of ethanol and 0·4 ml. was applied to a paper chromatogram which was subsequently developed in ethanol–aq. NH_3 soln. (sp.gr. 0·88)–water (6:3:1, by vol.). After spraying with acid molybdate reagent (Hanes & Isherwood, 1949) the paper was irradiated with u.v. light to locate the spots containing phosphorus and 1 cm. strips were scanned for radioactivity.

RESULTS

Hydrolysis of low-pressure monolayers of lecithin. When the purified enzyme solution was introduced into the aqueous phase below a pure [^{32}P]lecithin monolayer, at pressure below 30 dynes/cm. a loss of surface radioactivity was observed to occur. That this loss of surface counts coming from the film did truly represent hydrolysis of the lecithin was shown by taking the aqueous phase and subjecting it to paper chromatography. The radioactivity which appeared in the solution was exactly associated with an inactive glycerylphosphorylcholine marker which had been added to the solution (Fig. 3).

In films at a starting pressure of below about 16 dynes/cm. the loss of surface counts, representing the enzymic hydrolysis, was largely preceded by a progressive increase in both the surface potential and surface pressure (Fig. 4A). As the initial pressure of the film was increased above 16 dynes/cm. but below about 30 dynes/cm. the loss of radioactivity again followed changes in the surface pressure and potential which were the reverse of those occurring at lower pressures, i.e. both the

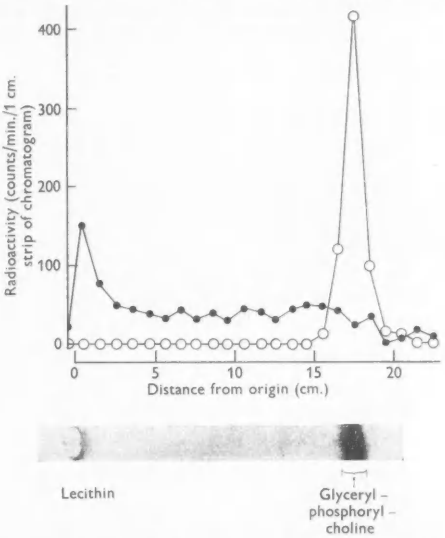

Fig. 3. Paper chromatography of the components containing ^{32}P in the aqueous phase (HCl at pH 3·3). ○, After enzymic hydrolysis of a low-pressure [^{32}P]lecithin film; ●, after an equivalent amount of [^{32}P]lecithin had been emulsified with HCl at pH 3·3 (no enzymic digestion). Lower chromatogram shows marker spots sprayed with reagent, etc., to reveal phosphate esters.

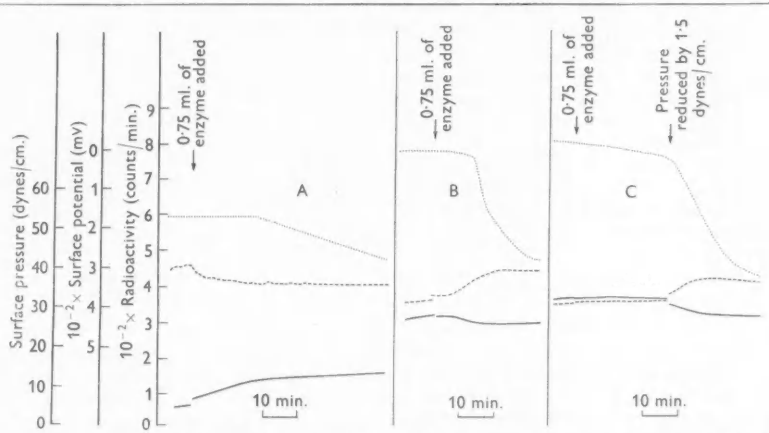

Fig. 4. A, B and C are separate experiments at different initial pressures showing the surface radioactivity, pressure and potential of a [^{32}P]lecithin monolayer undergoing enzymic hydrolysis. —, Surface pressure; - - - -, surface potential; . . . ., surface radioactivity (line evaluated from observations made every 18 sec.).

pressure and the potential decreased (Fig. 4B). If the starting pressure was increased to a level of about 30 dynes/cm. or above there were no changes in surface pressure or potential and no hydrolysis occurred. The sharpness of this pressure threshold is illustrated by the experiment shown in Fig. 4C, in which the reduction of the pressure of a pure lecithin film from 32·5 to 31 dynes/cm. is sufficient to cause the enzymic hydrolysis to begin. The threshold pressure at which hydrolysis ceased varied with individual enzyme preparations but generally lay in the range 28–33 dynes/cm.

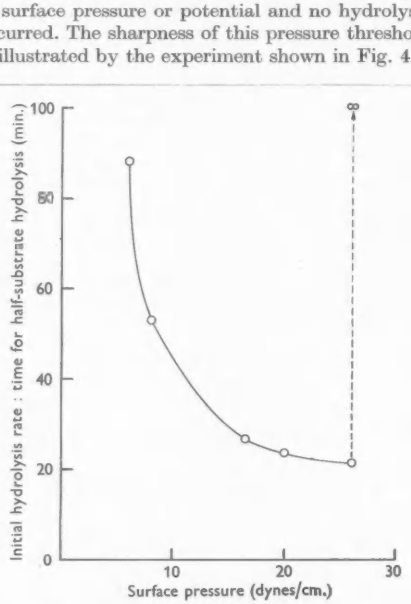

Fig. 5. Relationship between the surface pressure of a pure lecithin monolayer and its initial rate of enzymic hydrolysis.

It was observed (Figs. 4A, B) that the rate at which the film was hydrolysed increased with increasing initial starting pressures of the film up to a maximum just below the critical-pressure threshold. In Fig. 5 the time in minutes required for half the lecithin in the film to disappear has been plotted against the initial starting pressures of the lecithin films.

Hydrolysis of high-pressure mixed monolayers (dicetylphosphoric acid–lecithin). With a high-pressure film of lecithin, that is above about 30 dynes/cm., no change in hydrolysis or surface pressure or potential was observed (Fig. 6A). However, if progressive amounts of dicetylphosphoric acid were added to the lecithin monolayer the pressure threshold at which hydrolysis ceased could be raised. This was not observed if a positively charged amphipathic molecule was used instead of the dicetylphosphoric acid, e.g. heptadecylamine. Fig. 7 shows that there is a linear relationship between the molar percentage of dicetylphosphoric acid added and the pressure threshold at which hydrolysis ceased. With as much as 20–25 % of dicetylphosphoric acid added, hydrolysis occurred at the collapse pressure of the monolayer (45–50 dynes/cm.; Fig. 6B).

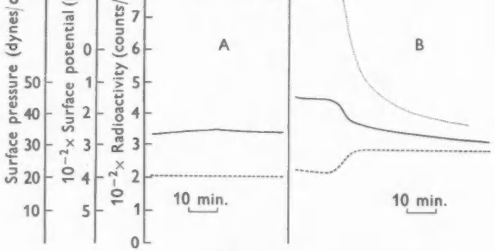

Fig. 6

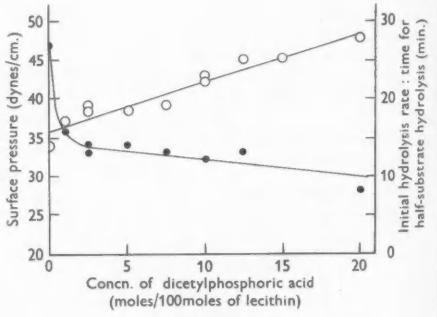
Surface pressure (dynes/cm.)
Initial hydrolysis rate : time for half-substrate hydrolysis (min.)
Concn. of dicetylphosphoric acid (moles/100moles of lecithin)

Fig. 7

Fig. 6. Recordings showing the surface radioactivity, pressure and potential of a [^{32}P]lecithin monolayer (A) and a [^{32}P]lecithin–25 % dicetylphosphoric acid mixed monolayer (B) at pressures above 30 dynes/cm. and in the presence of enzyme. —, Surface pressure; - - - -, surface potential; ...., surface radioactivity (line evaluated from observations made every 18 sec.).

Fig. 7. Relationship between the molar percentage of dicetylphosphoric acid in a lecithin monolayer and ○, the maximum pressure of the monolayer which allowed enzymic hydrolysis; or ●, the initial rate of hydrolysis when the initial pressure of the monolayer was the same as ○.

The reaction rate with films containing small amounts of dicetylphosphoric acid (1–2 %) was appreciably faster than that of the lecithin film alone; however, as the percentage of dicetylphosphoric acid was increased there was only a small further increase in this reaction rate (Fig. 7). With the technique of surface-radiation counting it would be expected that the rate be inversely proportional to the area per lecithin molecule. The resolution of the method, however, does not obviously reveal the hyperbolic nature of this relationship (Fig. 8).

Action of bivalent cations. If Ca^{2+} ions (3·6 mM) were added to the bulk phase below a monolayer of pure lecithin, enzymic hydrolysis occurred normally below pressures of about 30 dynes/cm. If a similar concentration of Ca^{2+} ions was added below a mixed monolayer of lecithin and dicetylphosphoric acid (20 %) at 45 dynes/cm. the surface potential increased, presumably due to counter-ion binding of the anion sites; at the same time no hydrolysis occurred when enzyme was added. If, however, the pressure of the mixed film was reduced, hydrolysis commenced at some 32 dynes/cm., a pressure which is near the threshold for hydrolysis of pure lecithin with or without Ca^{2+} ions present in the bulk phase.

Uranyl ions (UO_2^{2+}), which are noted for their high affinity for charged phosphate groups (Kruyt, 1949), were shown to inhibit the action of the enzyme on a lecithin–20 % dicetylphosphoric acid film (45 dynes/cm.) at a concentration of 1 mM. When the pressure of this film was reduced to 32 dynes/cm. or less the hydrolysis commenced.

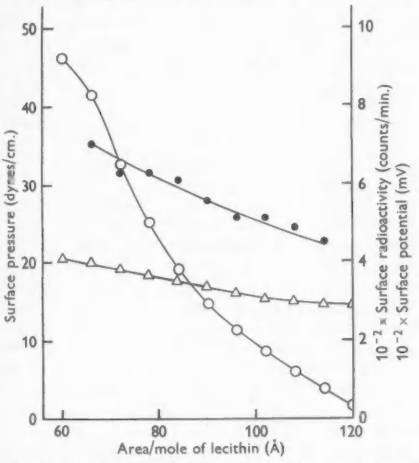

Fig. 8. Graphs showing the relationship between the area of a lecithin molecule in a monolayer and ○, surface pressure; ●, surface radioactivity; △, surface potential.

DISCUSSION

Previous experiments with collapsed films of [^{32}P]lecithin (Dawson & Bangham, 1959) had demonstrated that the presence of a net surplus of negative groups was a prerequisite for the enzymic attack of lecithin at its oil–water interface. With the methods used it was not possible to follow the hydrolysis of films other than those at their collapse pressure. With the present method, by the use of true monolayers it was possible to vary the surface pressure and to record both it and the surface potential continuously.

The results correlate with those obtained during earlier investigations on the relationship between the activity of the enzyme and the zeta potential of the lecithin particles of an emulsion (Bangham & Dawson, 1959*a*). It can be assumed that the surface of an emulsion particle would be equivalent to a monolayer of the same material at its collapse pressure. Thus in both cases a pure lecithin substrate was not attacked whereas lecithin containing a sufficient quantity of an anionic amphipathic molecule (dicetylphosphoric acid) was freely hydrolysed. It is pertinent that the enzymic hydrolysis of lecithin particles required the presence of a minimum percentage of dicetylphosphoric acid which was very similar in value to the amount required to initiate the hydrolysis of a monolayer at its collapse pressure.

With pure lecithin films which were hydrolysed only below a surface pressure of some 30 dynes/cm. it was not possible to demonstrate any change in surface potential by the addition of uni-univalent electrolyte (potassium chloride) to the aqueous phase. This indicates that at this pH (3·3) the lecithin is approximately isoelectric, and that, unlike monolayers above pressures of about 30 dynes/cm. excess of negative groups was not required for enzyme–substrate interaction. This is confirmed by the observation that hydrolysis of low-pressure lecithin or lecithin plus dicetylphosphoric acid films was not inhibited by the addition of bivalent cations or even by the addition of positive groups, e.g. heptadecylamine.

The increase of surface pressure observed when enzyme is introduced under a pure lecithin film at a pressure below 16 dynes/cm. would suggest that penetration or solution, or both, of proteins in the monolayer had occurred (Matalon & Schulman, 1949). However, it must be remembered that the enzyme preparation is not pure and starch–gel electrophoresis showed it to contain at least three components, one of which was electropositive at the pH (3·3) used. This means that the pressure rise observed is not necessarily due to penetration by enzyme.

It is also clear from Fig. 4 B that when enzyme is introduced below pure lecithin films above 16 dynes/

cm. the surface pressure and potential both fall. It is possible that these changes may be associated with the release of the first fatty acid from the lecithin molecule forming lysolecithin. This phospholipid would then be subsequently hydrolysed with the release of water-soluble glycerylphosphorylcholine and a further fatty acid. From the present results, which make use of an impure enzyme preparation, it is not possible to explain in what way the enzyme is reaching the fatty acid–ester bonds of the lecithin since the film is above the collapse pressure of most proteins and the conditions for electrostatic adsorption (Matalon & Schulman, 1949) are not evident.

With high-pressure films of pure lecithin it is apparent from all measurements that no interaction takes place between the added enzyme and the surface film. This is in accord with the observations of Fraser, Kaplan & Schulman (1955) on the adsorption of catalase by lecithin and other particles.

When negative anionic molecules are added with lecithin to form a mixed monolayer it is apparent that the negative groups encourage adsorption of the enzyme at pressures in excess of 30 dynes/cm. One possible explanation is that the negatively charged films would attract a positively charged region of the large enzyme molecule and thus assist its adsorption and penetration into the film (Bangham & Dawson, 1959*b*). This would be analogous to the adsorption of positively charged haemoglobin to a long-chain alkyl sulphate monolayer described by Matalon & Schulman (1949). Thus the greater the negative charge on the film, the greater would be its affinity for the enzyme, allowing hydrolysis to occur at ever-increasing surface pressures. The results suggest that the increase in the maximum pressure at which hydrolysis occurs is directly proportional to the percentage of dicetylphosphoric acid added.

The radioactivity of the lecithin–dicetylphosphoric acid mixed films suggested that, at the pressure at which hydrolysis commenced, the area per lecithin molecule was constant, and this could indicate that the crucial effect of the dicetylphosphoric acid molecules was one of surface dilution. However, if an equivalent surface dilution was obtained by using a positively charged long-chain alkylamine no hydrolysis occurred. This indicated that electrostatic conditions at the interface were of prime importance, a conclusion confirmed by the inhibitory action of bivalent cations at constant area per lecithin molecule.

SUMMARY

1. The enzymic hydrolysis of unimolecular films of yeast [^{32}P]lecithin by a purified preparation of a phospholipase B from *Penicillium notatum* has been studied.

2. The rate of the hydrolysis was measured continuously by recording the loss of surface radiation as the water-soluble [^{32}P]glycerylphosphorylcholine was stirred into the bulk aqueous phase. Simultaneously, continuous determinations were made of the surface pressure of the film and the potential between the air above the film and the aqueous phase.

3. The enzyme preparation attacked low-pressure films of lecithin (< approx. 30 dynes/cm.) and it was shown by paper chromatography that labelled glycerylphosphorylcholine was present in the aqueous phase. The rate of hydrolysis increased as the pressure of the lecithin film was increased up to a critical pressure, above which hydrolysis no longer occurred.

4. Addition of anionic amphipathic molecules (dicetylphosphoric acid) to the film allowed the lecithin to be hydrolysed at pressures above those at which a pure lecithin film was attacked.

5. There was a linear correlation between the threshold pressure above which the hydrolysis did not occur and the amount of dicetylphosphoric acid added to the lecithin: 20–25 molar % produced hydrolysis at the collapse pressure of the film.

6. The addition of calcium or uranyl counter ions to the aqueous phase inhibited the hydrolysis of high-pressure lecithin–dicetylphosphoric acid mixed films, but not that of low-pressure lecithin films.

7. It is concluded, tentatively, that the enzyme can penetrate into low-pressure films of lecithin, whereas with high-pressure films a negatively charged surface is required to assist penetration or adsorption or both.

REFERENCES

Adam, N. K. (1941). *The Physics and Chemistry of Surfaces*, 3rd ed., p. 23. Oxford University Press.

Bangham, A. D. & Dawson, R. M. C. (1959*a*). *Biochem. J.* **72**, 486.

Bangham, A. D. & Dawson, R. M. C. (1959*b*). *Proc. 7th European Soc. of Haematology, London. Acta haemat.* (in the Press).

Dawson, R. M. C. (1956). *Biochem. J.* **62**, 689.

Dawson, R. M. C. (1958*a*). *Biochem. J.* **68**, 352.

Dawson, R. M. C. (1958*b*). *Biochem. J.* **70**, 559.

Dawson, R. M. C. (1960). *Biochem. J.* **75**, 45.

Dawson, R. M. C. & Bangham, A. D. (1959). *Biochem. J.* **72**, 493.

Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.

Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957). *J. biol. Chem.* **226**, 497.

Fraser, M. J., Kaplan, J. G. & Schulman, J. H. (1955). *Disc. Faraday Soc.* **20**, 44.

Hanes, C. S. & Isherwood, F. A. (1949). *Nature, Lond.*, **164**, 1107.

Kruyt, H. R. (1949). *Colloid Science*, vol. 2, ch. 9. Amsterdam: Elsevier.

Matalon, R. & Schulman, J. H. (1949). *Disc. Faraday Soc.* **6**, 27.

Wilhemy, J. (1863). *Ann. Chim.* (*Phys.*), **119**, 177.

The Inactivation of a Bacteriophage by a Component of Papain

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It was found by Kalmanson & Bronfenbrenner (1943) that papain could reactivate phage PC which had been inactivated by antiserum and that the phage itself was unaffected by the enzyme. When this experiment was repeated with 'phage 3' in this Laboratory it was found that the phage was greatly inactivated. During an investigation of the mode of inactivation of this phage by papain the work of Kozloff, Lute & Henderson (1957) was published. These authors included crystalline papain in a list of agents which could inactivate coliphages T2 and T4 by damaging the tail tip, a part of the phage which must be intact for adsorption to host cells to occur. The agents they used had in common the property, recently described in papain by Johnston (1956), of breaking thiolester bonds. Kozloff *et al.* (1957) concluded that papain damaged the phage by breaking thiolester bonds which normally were responsible for the integrity of the tail in the active phage particle. That papain acts on the tails of both phage T4 and phage 3 seems adequately proven since both these phages require tryptophan for adsorption and both (Kozloff *et al.* 1957; Fildes & Kay, 1959) require tryptophan to be inactivated by papain. As adsorption involves only the tip of the phage tail then presumably papain attacks that part of the phage. However, as shown in this paper, the action of papain on phage 3 is not due to its thiolesterase activity.

MATERIALS AND METHODS

Bacteriophage and host bacteria. Phage 3 and its host *Bacterium coli* 518(3) have been described by Fildes & Kay (1959). The phage was isolated from Oxford sewage and, like the coliphages T2, T4 and T6, was found to contain the unusual pyrimidine hydroxymethylcytosine. It exists in two mutant forms, one of which requires tryptophan for adsorption to the host, whereas the other is largely independent. The latter was used in the work described in this paper. Stocks of the phage were prepared in an ammonia–glucose medium and purified by differential centrifuging as described by Kay (1959).

Measurement of the antiphage activity of papain. The papain preparations for testing were always made up in a buffer containing KH_2PO_4–Na_2HPO_4, pH 7·6, 0·033 M with respect to phosphate. This buffer is referred to as phosphate buffer in this paper. Serial dilutions (ratio 2:3 over the centre of the active range) of papain in this buffer were mixed with standard amounts of phage 3 (5×10^5/ml.) and incubated at 37° for 30 min. The total volume was 10 ml. DL-Tryptophan (0·1 mM) was added to enable the reaction between phage and papain to proceed at maximum rate, even though the phage was a mutant that was virtually independent of tryptophan. The effect of tryptophan on the action of papain on phage 3 was discussed by Fildes & Kay (1959). Controls without papain were set up and assayed for phage, as described by Fildes & Kay (1957), at the beginning, middle and end of the experiment and averaged to give the input. All the tubes were assayed for phage at 30 min. and the percentage inactivation was plotted against concentration of papain. The amount of papain required to destroy 50% of the input phage was calculated from the curves. Typical curves are given in Fig. 4.

Measurement of thiolesterase activity. Benzoylglycine thiol ethyl ester (BGTEE) was used as a substrate for thiolesterase. It was synthesized from benzoylglycine by the method described by Johnston (1956). In order to permit the thiolesterase of papain to act on BGTEE under conditions as similar as possible to those in which papain was tested against phage the tests were made in phosphate buffer (0·033 M), pH 7·6. The standard input of BGTEE was 5 μmoles in a volume of 1·5 ml. Methanol (10%, v/v) was included to maintain the BGTEE in solution. The papain to be tested, already in phosphate buffer, was diluted appropriately in the same buffer and added to the BGTEE. Thiolester was determined immediately and after 30 or 120 min. at 37° by the hydroxamic procedure of Lipmann & Tuttle (1945). The resulting coloured mixtures were centrifuged to remove the protein precipitates. The extinctions of the supernatants were measured in a Hilger Spekker absorptiometer with filters with a transmission maximum at 550 mμ. The extinction bore a linear relationship to the amount of BGTEE up to at least 8 μmoles.

Electrophoresis. Zone electrophoresis in a starch-grain bed 8 cm. × 30 cm. × 1 cm. deep at 4° was performed by a method similar to that described by Kunkel (1954). Potato starch (British Drug Houses Ltd.) was first washed in water and then equilibrated against three changes of the buffer chosen for the experiment. The sample of papain to be examined was incorporated into the starch bed near the anode end as it was known that all the components were positively charged at the pH chosen. Electrophoresis was carried out at 3–4 v/cm. as measured across the starch bed. The movement of the band was followed by taking filter-paper prints of the surface of the starch, drying the paper and staining in ethanolic 0·1% bromophenol blue, containing 4% (w/v) of mercuric chloride, for 1 min. The positions of the protein bands were revealed on washing the strips in 1% (v/v) acetic acid. When the bands had travelled

a suitable distance the starch bed was cut into sections and the protein extracted by suspension of the starch in 5 ml. of phosphate buffer followed by centrifuging and removal of the supernatant. This procedure was repeated twice; the extracts were pooled and finally filtered through Whatman paper no. 1 to remove the remaining starch grains. Microbial contamination was prevented by inclusion of a small crystal of thymol in the extracts.

Nitrogen determination. The protein-nitrogen content of the various samples studied was determined by the micro-Kjeldahl procedure of Campbell & Hanna (1937). A correction occasionally had to be made for nitrogen contributed by traces of ammonium sulphate remaining in fractions prepared by the use of this material.

Crude papain. All the papain used was obtained in a single batch from British Drug Houses Ltd. It was a fine light-brown powder and was presumably pulverized dried latex.

RESULTS

Antiphage activity of crude-papain extracts. Extracts of crude papain in phosphate buffer were tested against phage 3 at a wide range of dilutions. The phage and papain were allowed to react for 30 min., after which the amount of phage remaining was determined. A typical result is given in Fig. 1. It shows that the highest concentration of papain has little effect on the phage but that as the concentration is decreased the amount of phage destroyed rises to a maximum, when 93 % is lost, and then falls again. All extracts of papain, whether made in water, phosphate buffer or cysteine solution (provided that the last-named extract was dialysed before testing), showed this same type of curve, which suggests that they contained two active components, an inhibitor which was diluted out first and an antiphage material which was still active at high dilutions.

Effect of various agents on the antiphage extracts of papain. During the course of experiments to decide whether the proteolytic enzyme of papain was involved in the antiphage action it became clear that such agents as cysteine, ethylenediaminetetra-acetic acid (EDTA) and citrate, which activate the protease, inhibited the antiphage activity of papain. Cysteine, depending on the concentration relative to that of the papain, could block the antiphage component and the hypothetical inhibitory component. In Fig. 1 are two curves showing the effect of cysteine. For one, papain was titrated against phage in the presence of constant (0·033 M) cysteine. The antiphage activity was reduced but the inhibitory effect was also diminished since more phage was lost in the highest papain concentration with cysteine than without. For the second curve, papain and cysteine at an initial concentration of 0·025 M were diluted together. The antiphage activity was largely unaffected and was not suppressed at high concentrations of papain. It appears therefore that a certain ratio of cysteine to papain can block the hypothetical inhibitor of antiphage but that higher ratios block the antiphage as well. The effect of citrate and EDTA on the antiphage activity of papain is shown in Fig. 2. The amount of papain, in this case an ammonium sulphate fraction, was that which gave the maximum inhibition of phage when tested in phosphate buffer. This was kept constant while the citrate and EDTA were varied. The

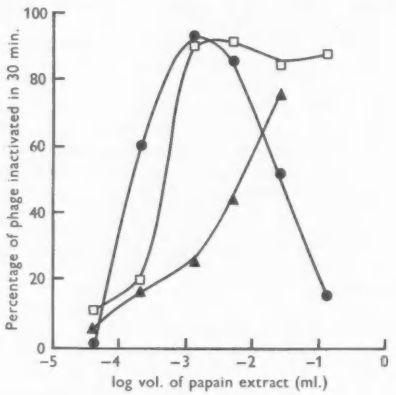

Fig. 1. Inactivation of phage 3 by papain. An extract of 4 g. of papain in 100 ml. of phosphate buffer (0·033 M), pH 7·6, was tested in fivefold serial dilutions against phage 3 (5×10^5/ml.) at 37° for 30 min. in phosphate buffer (●), in the presence of constant (0·033 M) cysteine (▲) and in cysteine diluted with the papain from 0·025 M (□).

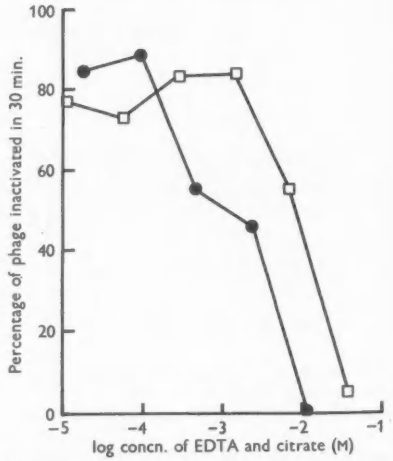

Fig. 2. Effect of citrate (□) and EDTA (●) on the inactivation of phage 3 by a constant amount of papain.

amount of phage inactivated is plotted against the concentration of these substances. Both EDTA and citrate abolish the antiphage activity if added in the appropriate concentration, EDTA being rather more active than citrate. The three agents tested above, cysteine, EDTA and citrate, have in common the property of sequestering metal ions and their action suggests that some metal is an essential component of the antiphage system. All the tests have been done in 0·033 M-phosphate buffer, and it is known that this, too, is capable of binding some metal ions and effectively removing them from the system. It was therefore of importance to test whether phosphate itself was affecting the antiphage activity of papain, and for this purpose two sets of reactants, phage, tryptophan and papain, were prepared, one in the usual phosphate buffer and the other in 0·033 M-sodium chloride. Dilutions of papain were then tested against phage, with the results given in Fig. 3. The antiphage activity of papain is greatly reduced in the absence of phosphate, which suggests that some inhibitory ions normally present in the reaction mixture are sufficiently reduced in concentration by the phosphate to permit the papain to inactivate the phage. However, taking other observations into account, together with the inhibitory effect of citrate etc., it appears that a particular balance between the papain and metal ions must be set up for papain to exert its maximum destructive effect on phage. It has not yet been possible to clarify this situation and to determine what metals are involved in the papain–phage system, and since the main object of this paper is to consider the thiolesterase of papain and its role in phage-inactivation the matter of the metals and hypothetical inhibitor will not be examined further.

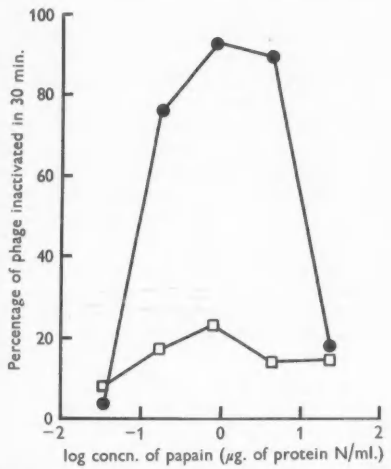

Fig. 3. Effect of phosphate on the antiphage activity of papain. An extract of papain was tested in fivefold dilutions against phage 3 in phosphate buffer (0·033 M) (●) and in NaCl (0·033 M) adjusted to pH 7·6 (□).

Effect of crystalline papain on phage. Papain was crystallized by the method of Kimmel & Smith (1954), starting from dried powdered latex. In this procedure an extract of latex in 0·04 M-cysteine is prepared. This is adjusted to pH 9·0 to remove an inactive component, treated with 0·4 saturated ammonium sulphate to precipitate the protease, which is then crystallized from 0·02 M-cysteine solution. After two recrystallizations from sodium chloride solution the crystals were centrifuged down, dissolved in a little water and dried from the frozen state and stored over phosphorus pentoxide. The antiphage activity of this material dissolved in phosphate buffer was tested by the standard procedure, and the results, together with those of the solution before crystallization and the combined mother liquors after two recrystallizations, are shown in Table 1. It is clear that the antiphage activity is not in the crystalline material.

Thiolesterase of crystalline papain. Extracts of crude-papain latex and crystalline mercuripapain have been found by Johnston (1956) to hydrolyse the thiolester bonds of BGTEE and thiol *iso*propyl ester. The crystalline papain as prepared above and found to be inactive against phage was then tested for thiolesterase against BGTEE. Thiolesterase activity was found, as shown in Table 2.

Table 1. *Inactivation of phage* 3 *by fractions of papain*

Ammonium sulphate fraction before crystallization		Protease fraction (twice recrystallized)		Mother liquors	
Protein N (μg./ml.)	Inactivation (%)	Protein N (μg./ml.)	Inactivation (%)	Protein N (μg./ml.)	Inactivation (%)
53·4	17	450	0	25·6	36
10·7	49	90	0	5·1	76
2·1	57	33·4	0	1·0	25
0·43	32	6·7	0	0·2	2
0·85	4	1·3	0	0·04	0

Fractionation of papain. As it had been shown that the crystallizable component of papain did not contain the antiphage activity but did have thiolesterase activity, doubts arose as to the likelihood of the antiphage component having thiolesterase activity, although this was still a possibility. It was therefore decided to fractionate the ammonium sulphate-soluble part of the cysteine extract of latex which had been discarded during the isolation of crystalline papain. Further fractions were prepared by raising the ammonium sulphate concentration to 50, 70 and 100% saturated. Antiphage activity and thiolesterase were found in all of these fractions but were most marked in the 50% and 70% saturated ammonium sulphate precipitates. However, it became obvious that the separation of thiolesterase and antiphage activity was not feasible by this procedure, and so another method, electrophoresis, was tried. A preliminary test, in which the 40–70% saturated ammonium sulphate fraction was examined in the Antweiler microelectrophoresis apparatus, showed that at pH 5·0, 7·0 and 9·0, in acetate, phosphate and borate buffers (0·02 M with respect to these ions), the papain divided into several components, all of which were positively charged. The separation appeared to be most complete at pH 5·0, and so it was decided to fractionate papain by preparative electrophoresis at this value. The starch-grain-bed procedure was adopted. The starting material was a 40–70% saturated ammonium sulphate precipitate prepared from the mother liquors left after removal of the 40% saturated ammonium sulphate precipitate in the Kimmel & Smith (1954) procedure for crystallizing the protease. The precipitate was redissolved in sodium acetate–acetic acid buffer, pH 5·0 (0·02 M-acetate), and incorporated (22 mg. of protein nitrogen) into the starch layer. After electrophoresis at 3–4 v/cm. for 24 hr. it was found that the leading edge of the protein band, which was about 8 cm. long, had reached to within 1 cm. of the end of the starch bed and was divided into a sharp leading band followed by a second diffuse band. The protein bands were then removed and cut into four sections, one of which contained the

Table 2. *Hydrolysis of benzoylglycine thiol ethyl ester by crystalline papain*

Input of thiolester was 5 μmoles in a total volume of 1·5 ml. of phosphate buffer (0·033 M), pH 7·6, containing 10% (v/v) of methanol. The mixture was kept at 37° for 30 min.

Crystalline papain (μg. of protein N/ml.)	Thiolester hydrolysed (% of input)
38·0	96·5
19·0	75·0
9·5	35·5
4·8	17·5
None	7·0

Table 3. *Electrophoretic fractionation of papain at* pH 5·0

The fraction of papain soluble in 40% saturated ammonium sulphate but precipitated by 70% saturated ammonium sulphate was subjected to electrophoresis in starch in acetate buffer, pH 5·0. The protein band was divided into four parts, numbered 1 to 4 from the anode end, and tested for antiphage activity and for thiolesterase activity.

Fraction tested	Protein N in test (μg./ml.)	Thiolester hydrolysed (%) 30 min.	Thiolester hydrolysed (%) 120 min.	Protein N to inactivate 50% of phage (μg./ml.)
40–70% Ammonium sulphate ppt.	248	100	—	0·08
	124	96	—	
	49	60	—	
	32	—	60	
Fraction 1	67	—	0	7·5
	50	0	—	
	25	0	—	
	10	0	—	
Fraction 2	148	—	100	0·80
	111	96	—	
	56	64	—	
	33	18	—	
Fraction 3	110	—	100	0·035
	82	86	—	
	41	53	—	
	24	8	—	
Fraction 4	32	—	0	0·0045
	24	0	—	
	12	0	—	
	7	0	—	

well-defined frontal band and the rest were equal parts of the remaining diffuse band. They were numbered 1 to 4 from the anode end. The protein in each fraction was extracted with three lots of 5 ml. phosphate buffer, pH 7·6 (0·033 M), by centrifuging and tested for protein-nitrogen content, antiphage activity and thiolesterase activity. The results are given in Table 3 and Fig. 4.

Fig. 4 shows the relationship between protein-nitrogen concentration and phage inactivated in 30 min. From it the amounts of protein nitrogen required to destroy 50 % of the phage in 30 min. were obtained and included in Table 3. It appears that all fractions contained some antiphage activity, but that this lay predominantly in fraction 4, by comparison with which the others had small activities in the approximate ratio 1600:180:8:1 in the amounts of protein nitrogen required to inactivate 50 % of the phage. Thiolesterase was found only in fractions 2 and 3. None could be detected in fraction 4 even at the highest concentration and longest time (2 hr.) tested. It was not possible to lengthen the duration of the test owing to the instability of the thiolester, which decreased by 12–15 % in 2 hr. in the controls. Nevertheless, the above results show that papain could be divided by electrophoresis into one fraction (no. 4) which contained a very active antiphage and no thiolesterase and other fractions which contained thiolesterase and antiphage material of a much lower activity. This is taken to mean that papain contains an antiphage component which is not a thiolesterase. Further electrophoretic fractionations were made at pH 7·6 and 4·0. At pH 7·6, which was maintained in 0·02 M-phosphate buffer, a less-satisfactory separation of antiphage activity of thiolesterase was obtained. No clearly defined front band was observed, but on extraction from the starch the material at the front of the diffuse protein band showed the highest antiphage activity and the lowest thiolesterase activity. The

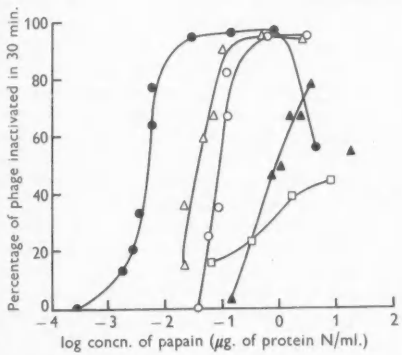

Fig. 4. Antiphage activity of a 0·4–0·7 saturated ammonium sulphate fraction of papain (○) compared with that of fractions 1 (□), 2 (▲), 3 (△) and 4 (●), prepared at pH 5·0 by zone electrophoresis i n starch.

Table 4. *Electrophoretic fractionation of papain at* pH 4·0

The fraction of papain soluble in 40 % saturated ammonium sulphate but precipitated by 70 % saturated ammonium sulphate was subjected to electrophoresis in starch in formate buffer, pH 4·0. The protein band was divided into four parts numbered 1 to 4 from the anode end and tested for antiphage activity and for thiolesterase activity.

Fraction tested	Protein N in test (μg./ml.)	Thiolester hydrolysed in 120 min. (%)	Protein N to inactivate 50 % of phage (μg./ml.)
40–70 % Ammonium sulphate ppt.	32	88	0·05
	19	46	
	13	26	
	6	11	
Fraction 1	73	41	2·5
	32	4	
	19	0	
	13	0	
Fraction 2	96	100	1·0
	32	34	
	19	0	
	13	0	
Fraction 3	32	0	0·05
	19	0	
	13	0	
Fraction 4	45	0	0·016
	32	0	
	19	0	
	13	0	

separation at pH 4·0 was performed in sodium formate–formic acid buffer (0·02 M-formate). Again a clear separation of antiphage and thiolesterase activity was obtained. The main protein band was preceded by a minor but well-defined band. This proved on extraction to contain the most active antiphage material and, as at pH 5·0, no thiolesterase. The results are given in Table 4.

Effect of cysteine on thiolesterase and on phage 3. Johnston (1956) observed that cysteine, when used in an attempt to activate the thiolesterase of crystalline papain, was itself able to destroy the benzoylglycine thiol ethyl ester used as a substrate. This result has been confirmed during the present work, as is shown in Table 5. Cysteine in varying amounts was incubated with BGTEE (3·4 μM) in phosphate buffer (0·033 M), pH 7·6, containing methanol (10 %, v/v) to maintain the BGTEE in solution. After 30 min. at 37° the amount of BGTEE remaining was determined. It can be seen that in an equimolar mixture of cysteine and BGTEE, 57 % of the BGTEE has been lost by reaction with the cysteine. Larger amounts of cysteine destroy 82 % of the BGTEE in 30 min. The reaction, which is of a type described by Strecker, Mela & Waelsch (1955), is said to involve a sulphur-to-sulphur transfer of the acyl amino acid group of the thiol ethyl ester to the thiol group of cysteine, followed by a rearrangement during which the acyl group migrates to the nitrogen of the cysteine. The *N*-acyl cysteine does not form a hydroxamic acid under the conditions of the analysis.

The activity of phage 3 was not affected by treating for 30 min. at 10^5 particles/ml. in 0·033 M-phosphate buffer, pH 7·6, with cysteine varying from 0·05 to 0·002 M.

DISCUSSION

The results described confirm the discovery of Johnston (1956) that papain contains a component capable of splitting the thiolester bonds of benzoylglycine thiol ethyl ester. This component, known as a thiolesterase, occurs in the crystallizable protease fraction as isolated by the procedure of Kimmel & Smith (1954) and is probably identical with it. Kozloff *et al.* (1957) found that a commercially produced crystalline papain could inactivate coliphages T2 and T4 under conditions in which the thiolesterase would be active but the protease would be inactive. They believed that the papain inactivated the phages by breaking thiolester bonds in their tails. In an attempt to confirm this result with phage 3, which is somewhat similar to T4, it was found that whereas crude papain caused rapid inactivation, crystalline papain was without effect. The antiphage activity resided in the fractions of papain discarded during crystallization of the protease. Zone electrophoresis in starch showed that the majority of the antiphage activity travelled as an isolated band ahead of the main protein band. The material in this band was found to possess powerful antiphage activity but indetectable thiolesterase activity. It is concluded therefore that the thiolesterase is not responsible for the action of papain on phage 3. Presumably thiolester bonds, if they exist in phage 3, are not available to the thiolesterase of papain nor are they susceptible to the action of cysteine, which rapidly destroys thiolester bonds in BGTEE but has no effect on the activity of phage 3. The antiphage component of papain is being investigated further.

The principal difference between the results of Kozloff *et al.* (1957) and those described in the present paper is that whereas the former support the view that papain inactivates phage T2 by breaking thiolester bonds the latter show that the inactivation of phage 3 by papain is unlikely to be due to its thiolesterase. At present it has not been resolved whether this difference is due to different phages or to different experimental conditions.

Table 5. *Destruction of thiolester by cysteine*

Constant amounts of BGTEE (3·4 mM) were incubated at 37° in phosphate buffer (0·033 M), pH 7·6, with the indicated amounts of cysteine in a total volume of 1·5 ml., including 10 % (v/v) of methanol.

Cysteine (mM)	Thiolester lost in 30 min.
33	90
13	91
6·7	90
3·4	66
1·3	34
0·67	25
None	9

SUMMARY

1. A bacteriophage, phage 3, originally isolated from Oxford sewage, is inactivated by dilute extracts of papain.

2. The inactivation is blocked by cysteine, citrate and ethylenediaminetetra-acetic acid, which suggests that a metal might be involved in the system.

3. The protease of papain, when crystallized by the procedure of Kimmel & Smith (1954), was found to possess thiolesterase activity as shown by Johnston (1956).

4. The thiolesterase has no effect on phage 3 although Kozloff *et al.* (1957) have found that the somewhat similar phages T2 and T4 are inactivated by the thiolesterase of crystalline papain.

5. The antiphage component of crude papain has been separated by zone electrophoresis and found to have no thiolesterase activity.

The authors wish to thank Miss Ann Freeman and Miss Emmie Tschudin for technical assistance.

REFERENCES

Campbell, W. R. & Hanna, M. I. (1937). *J. biol. Chem.* **119**, 1.
Fildes, P. & Kay, D. (1957). *Brit. J. exp. Path.* **38**, 563.
Fildes, P. & Kay, D. (1959). *Brit. J. exp. Path.* **40**, 71.
Johnston, R. B. (1956). *J. biol. Chem.* **221**, 1037.
Kalmanson, G. M. & Bronfenbrenner, J. (1943). *J. Immunol.* **47**, 387.
Kay, D. (1959). *Biochem. J.* **73**, 149.
Kimmel, J. R. & Smith, E. L. (1954). *J. biol. Chem.* **207**, 515.
Kozloff, L. M., Lute, M. & Henderson, K. (1957). *J. biol. Chem.* **228**, 511.
Kunkel, H. G. (1954). *Meth. biochem. Anal.* **1**, 141.
Lipmann, F. & Tuttle, L. C. (1945). *J. biol. Chem.* **159**, 21.
Streecker, H. J., Mela, P. & Waelsch, H. (1955). *J. biol. Chem.* **212**, 223.

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Parapepsinogen II: The Zymogen of Parapepsin II

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The isolation and characterization of two new enzymes, parapepsins I and II, from crude porcine pepsin has already been described (Ryle & Porter, 1959).

In seeking to determine whether these enzymes might be derived from pepsinogen or whether they arose from other precursors partly purified extracts of porcine fundic mucosa have been chromatographed on diethylaminoethylcellulose and a zymogen for parapepsin II has been found. The isolation and some of the properties of this zymogen are described in this paper.

EXPERIMENTAL

Abbreviations. The following abbreviations will be used throughout this paper: diethylaminoethyl, DEAE; haemoglobin, Hb; acetyl-DL-phenylalanyl-L-di-iodotyrosine, APD; 1-fluoro-2:4-dinitrobenzene, FDNB; dinitrophenyl, DNP.

Instruments. Extinction coefficients were determined with either the Hilger Uvispek or a Unicam spectrophotometer.

Determinations of pH were made either with an E.I.L. direct-reading pH meter or a Cambridge pH meter, the meters being standardized against buffer solutions made from Cambridge buffer tablets.

Buffer solutions. Except where otherwise stated buffer solutions were made from sodium salts. Their concentrations are stated with respect to the anionic component.

Activity determination with haemoglobin. Assays of activity against haemoglobin were performed in duplicate by a method based on that of Northrop, Kunitz & Herriott (1948) as described by Ryle & Porter (1959). In the earlier part of the work, when zymogens were assayed 0·1 ml. of the zymogen solution in 0·02 M-phosphate buffer, pH 6·9, was mixed with 0·1 ml. of 0·035 N-HCl and allowed to stand at 35·5° for 10 min. to allow activation to occur before addition of 1 ml. of acid-denatured haemoglobin (2 %, w/v). Later it was found that no significant difference appeared in the assay of either pepsinogen or parapepsinogen II if this activation period were omitted, and 0·2 ml. samples were then assayed by the addition of 1 ml. of acid-denatured

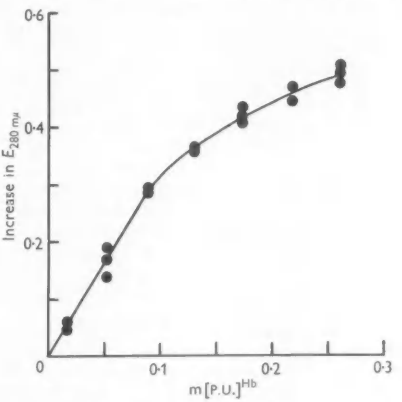

Fig. 1. Standard curve for parapepsin II. A solution (0·2 ml.) of enzyme in 0·1 M-acetate, pH 5·6, was incubated with 1 ml. of acid-denatured haemoglobin (4 vol. of 2·5 % Hb + 1 vol. of 0·39 N-HCl) for 10 min. at 35·5°. The reaction was stopped with 5 ml. of 4 % (w/v) trichloroacetic acid. Units were chosen as described in the text.

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haemoglobin [0·8 ml. of 2·5% (w/v) Hb + 0·2 ml. of 0·315 N-HCl]. In all the haemoglobin assays the total amount of acid used was such as to give a digestion mixture of pH 1·7.

The increments of *E* found for pepsinogen were converted into milliproteolytic units (m[P.U.]^{Hb}) by reference to a standard curve obtained with a preparation of crystalline pepsin which had been standardized by the original method of Anson (1938). For parapepsinogen II a new standard curve, prepared with a solution of parapepsin II, was used (Fig. 1). The units were chosen so that with an increment of *E* of 0·28 the activities determined by the two curves are equal (0·0855 m[P.U.]). Since the assays previously performed (Ryle & Porter, 1959) were as far as possible restricted to this region the difference between the units found with the curve for parapepsin II and those obtained with the pepsin curve is small. With the new curve assays have usually been restricted to the range 0·05–0·12 m[P.U.]^{Hb}, where the reproducibility is maximal.

*Activity determinations with acetyl-*DL*-phenylalanyl-*L*-di-iodotyrosine.* Determinations of activity against APD were carried out on the zymogens in a manner similar to that used for the enzymes by Ryle & Porter (1959). At first, as in the haemoglobin assay, a 10 min. period was allowed for activation to occur, but after it had been found that omission of this period made no difference to the activity the assays were performed without previous activation. Instead of the ninhydrin reagent containing acetate and citrate buffers and stannous chloride the modified reagent of Moore & Stein (1954) was used at first, and was found to give higher colour yields than the stannous chloride reagent. It was later found that the small amount of phosphate present in the assay mixtures did not cause precipitation of the stannous chloride and the reagent containing this reducing agent has also been used. To 0·5 ml. of solution to be assayed 0·25 ml. of 0·088 N-HCl is added at 35·5°, followed 15 sec. later by 0·25 ml. of 2 mM-APD in 0·01 N-NaOH to give a solution pH 2·0. After 20 min. at 35·5° the digestion is stopped by the addition of 1 ml. of ninhydrin reagent and the colour is developed and read in the usual way.

Milk-clotting activity. Determinations of milk-clotting activity were made as in the earlier work (Ryle & Porter, 1959) except that, following Berridge (1955), the milk solution contained 0·01 M-$CaCl_2$. Under these conditions parapepsin II clotted the milk more rapidly than it did when $CaCl_2$ was not added so that this enzyme showed a ratio [P.U.]$^{Ren.}$:m[P.U.]^{Hb} higher than 0·47, found in the absence of added calcium. Since these activity ratios were found to be somewhat variable the ratio for parapepsin II was determined at the same time as that for the zymogen when a comparison was to be made. One [P.U.]$^{Ren.}$ is the amount of enzyme which will clot 11 ml. of enzyme–milk mixture in 1 min. at 35·5°.

Preparation of crude pepsinogen solution. Pig stomachs were collected, rinsed and packed in ice for transport to the Laboratory. The fundic parts of the mucosae were removed and either used at once or frozen at −10° for storage. No difference was observed between preparations made from mucosae used at once or from those stored for several weeks.

The pepsinogen was extracted and partly purified by the method of Herriott (1948) up to the stage of precipitation of the zymogen with 0·68-saturated $(NH_4)_2SO_4$, filtration and re-solution in water (about 50 ml./100 g. of mucosa). The recovery of activity up to this step has been close to that found by Herriott (80%) and close to 100% in preparations made from single mucosae.

The solutions thus obtained were concentrated by ultrafiltration under 60 cm. Hg pressure through $\frac{1}{32}$ in. dialysis tubing (Visking Corp., obtained from Viscose Ltd., 40, Chancery Lane, London, W.C. 2) supported by a sleeve of silk net and dialysed against 0·02 M-phosphate buffer, pH 6·9.

Chromatography of crude pepsinogen. The dialysed solutions were fractionated by ion-exchange chromatography on columns of DEAE-cellulose (Peterson & Sober, 1956) in 0·02 M-phosphate buffer, pH 6·9, with a rising gradient of NaCl concentration obtained by passing a solution containing 0·45 M-NaCl and 0·02 M-phosphate buffer, pH 6·9, into a mixing vessel of fixed volume containing 0·02 M-phosphate buffer, pH 6·9.

The contents of the tubes from the active peaks were suitably pooled, concentrated by ultrafiltration through Visking $\frac{1}{32}$ in. tubing under 60 cm. Hg pressure, dialysed against distilled water and freeze-dried. These operations were all carried out at about 2° and solutions were kept saturated with toluene.

Nitrogen determination. Determination of total nitrogen in solutions of parapepsin II and parapepsinogen II were made by digestion with H_2SO_4 and estimation by ninhydrin of the NH_3 formed (Jacobs, 1959).

Determination of free amino groups. *N*-Terminal amino acid residues and lysine residues were determined by the fluorodinitrobenzene method [details of the techniques used are described by Porter (1957)]. Duplicate samples of the protein were allowed to react with FDNB in the presence of trimethylamine and, after removal of excess of FDNB and dinitrophenol by ether extraction, were hydrolysed in 5·7 N-HCl for 20 hr. at 105° under N_2; to one sample known amounts of DNP-serine and ε-DNP-lysine were added before hydrolysis so that correction could be made for the breakdown of these DNP-amino acids during the hydrolysis. The ether-soluble DNP-amino acids were separated by chromatography on paper in *tert.*-amyl alcohol (Blackburn & Lowther, 1951), estimated by their *E* at 360 mμ in 1% (w/v) $NaHCO_3$ soln. and, where necessary, had their identities confirmed by paper chromatography in phosphate buffer (Levy, 1954).

Portions of the aqueous fraction of the hydrolysates were chromatographed in butanol–acetic acid–water (1260:200:540, by vol.) on Whatman no. 3 MM paper, together with standard amounts of ε-DNP-lysine. The yellow spots were cut out, eluted with 5 ml. of N-HCl at 60° for 15 min., and the amounts of ε-DNP-lysine were determined by *E* at 350 mμ.

Analysis in the ultracentrifuge. The work with the ultracentrifuge was performed by Dr P. A. Charlwood of this Institute with a Spinco model E centrifuge. Determinations of molecular weight [by the method of Archibald (1947) as used by Charlwood (1957)] and of sedimentation coefficient were made in phosphate buffer, pH 6·9, *I* 0·20 (1·47 g. of $NaH_2PO_4,2H_2O$, 4·85 g. of $Na_2HPO_4,12H_2O$ and 11·18 g. of KCl/l.).

RESULTS

Chromatographic fractionation of crude pepsinogen. Fig. 2 shows a chromatogram of about six-sevenths of the crude pepsinogen obtained from a

single mucosa, containing potential activity against haemoglobin equivalent to about 870 mg. of pepsin. The large active peak has activity against both haemoglobin and acetyl-DL-phenylalanyl-L-di-iodotyrosine and is presumably pepsinogen. This material has not been studied further. The peak emerging at 4·4 l. after the application of the gradient shows activity against haemoglobin but little against the synthetic substrate. Because of this specificity, and the apparent stability of the activity at pH 6·9 (at which pH parapepsin II undergoes inactivation), it was tentatively concluded that the material in this peak was the zymogen of parapepsin II, parapepsinogen II. Further results have supported this conclusion.

The recovery of enzyme activity against haemoglobin from this column was 90% and the recovery of E at 280 mμ was 100%. The parapepsinogen II accounts for 7% of the total activity against haemoglobin.

Specific activity of parapepsin II and parapepsinogen II. Freeze-dried samples of the enzyme have had the specific activity $[\text{P.U.}]^{\text{Hb}}_{\text{mg. of N}}$ 0·31–0·34. The specific activity of the zymogen has varied from 0·28 to 0·32 $[\text{P.U.}]^{\text{Hb}}_{\text{mg. of N}}$.

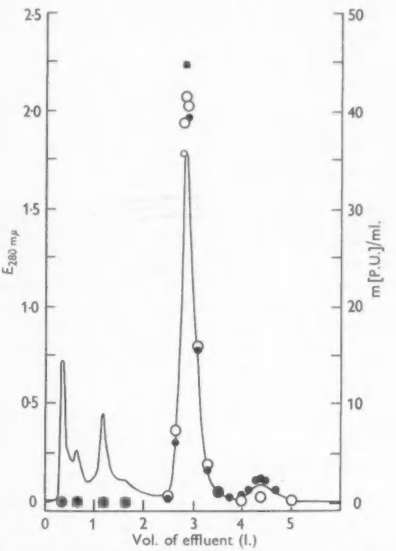

Fig. 2. Chromatography of partly purified pepsinogen, 20$[\text{P.U.}]^{\text{Hb}}$ in 28 ml., on a 5 cm. × 26 cm. column of DEAE-cellulose in 0·02 M-phosphate, pH 6·9. The gradient was 0–0·45 M-NaCl in the same buffer with a mixing vessel of 2240 ml. ●, Activity against Hb; ○, activity against APD. For clarity experimental points have been omitted from the curve of E at 280 mμ and the curves of activity against Hb and APD have not been drawn.

Stability of parapepsinogen II at pH 6·9. At 25° and pH 6·9 parapepsin II loses about half its activity in 1 hr. (Ryle & Porter, 1959). During the handling of parapepsinogen II solutions at this pH in the cold no definite losses of activity were observed and further experiments showed that solutions of the zymogen (4·5 and 2·5 m$[\text{P.U.}]^{\text{Hb}}$/ml.) in 0·02 M-phosphate buffer, pH 6·9, retained their potential activity for days at room temperature.

Chromatographic behaviour of activated parapepsinogen II. When parapepsinogen II which had been allowed to stand for 5 min. at pH 2·0 at room temperature was chromatographed on a column of DEAE-cellulose in 0·1 M-acetate buffer, pH 4·0, with rising NaCl gradient, an active peak emerged in the position parapepsin II was expected to occupy. Similarly, when parapepsinogen II which had been allowed to stand for 10 min. at pH 2·0 at room temperature was mixed with parapepsin II only one active peak appeared on the chromatogram, in the position of parapepsin II. This peak accounted for 65% of the activity applied, or 112% of the activity of the parapepsin II, so that both activated zymogen and enzyme must have been present in the single peak found. The recovery of parapepsin II from this type of column has usually been about 70–80%.

Study of parapepsinogen II in the ultracentrifuge. In a single run, the molecular weight found (the mean of five values determined at different times) was 40 500, and the sedimentation coefficient was 3·36 s at a concentration of 0·7%. The material showed only a single symmetrical sedimenting boundary.

Free amino groups in parapepsinogen II. In a preliminary experiment performed to determine which amino acid was *N*-terminal in parapepsinogen II, but in which internal breakdown controls were not used, the quantities of DNP-amino acids found per mole of protein, corrected for breakdown during hydrolysis by the figures of Porter (1957), were 0·9 mole of DNP-serine and about 10 moles of ϵ-DNP-lysine. Only very faint traces of other DNP-amino acids were found.

In a second experiment with a different batch of zymogen, the breakdown of DNP-serine and ϵ-DNP-lysine was determined by internal controls. The corrected amounts of these DNP-amino acids found, per 40 500 g. of protein, were: DNP-serine 0·9 mole, ϵ-DNP-lysine 8·9 moles. In addition, about 0·2 mole of fast-running DNP-amino acids, shown by chromatography in phosphate buffer to be mainly DNP-phenylalanine, were found.

Activation of parapepsinogen II. In the assay with haemoglobin it was found that a preliminary

period of incubation of parapepsinogen II at pH 2·0 and 35·5° for 5 or 10 min. in the absence of substrate did not increase the extent of digestion of haemoglobin subsequently added. At pH 1·7, therefore, the zymogen must be very rapidly activated.

For this reason it was not possible to follow the activation of the zymogen by the haemoglobin assay and so the assay by milk-clotting activity was used. This activity is measured at pH close to 5·6, and it was found that quantities of zymogen ten times as great as those which, after complete activation, would clot milk in 1·5 min., only brought about clotting in about 1 hr. The activation of the zymogen at pH 5·6 was therefore sufficiently slow to allow the milk-clotting method to be used to follow the conversion of zymogen into enzyme.

A solution of parapepsinogen II in 0·02 M-phosphate buffer, pH 6·9, was diluted tenfold with 0·1 M-acetate buffer, pH 4·0, at 35·5°, and the milk-clotting activities of samples taken at intervals were determined. The pH of the activation mixture, determined by the glass electrode at the end of the experiment, was 4·1. Fig. 3 shows that the activation of the zymogen follows first-order kinetics. However, the ratio $[P.U.]^{Ren.}:m[P.U.]^{Hb}$ for the activation mixture at 60 min. was only about 0·5, whereas the ratio for parapepsin II determined on the same milk substrate was 0·77. A portion (1 ml.) of the activation mixture was added to 0·028 ml. of N-HCl to reduce the pH to 2·0, and allowed to stand for 5 min. at 35·5°. It was then diluted tenfold with 0·1 M-acetate, pH 4·0. The milk-clotting activity of this acid-treated solution, calculated back to the concentration of the activation mixture, is shown in Fig. 3. The ratio $[P.U.]^{Ren.}:[P.U.]^{Hb}$ was now 0·8. Fig. 4 shows the course of the activation at pH 4·7. The kinetics here are not simply first-order or autocatalytic but appear to lie somewhere between.

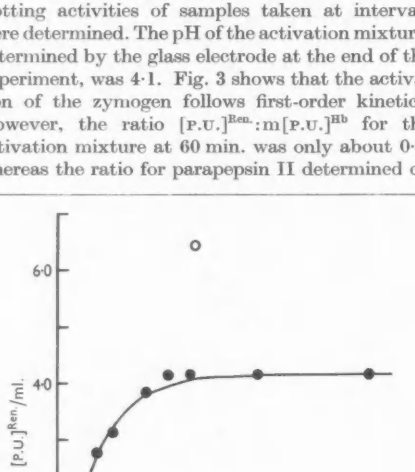

Fig. 3. Activation of parapepsinogen II at pH 4·1 and 35·5°. The activation mixture contained 1 ml. of a solution (3 mg./ml.) of parapepsinogen II in 0·02 M-phosphate, pH 6·9, and 9 ml. of 0·1 M-acetate, pH 4·0. ●, Milk-clotting activity; ○, milk-clotting activity after 5 min. incubation at pH 2·0 and 35·5°. The curve is the theoretical curve for a first-order reaction, K 0·06, taking the initial concentration of zymogen as 4·17 potential $[P.U.]^{Ren.}$/ml.

Mixed chromatogram of parapepsin II and parapepsinogen II. Fig. 5 shows the result of chromatographing about 10 mg. each of parapepsin II and parapepsinogen II at pH 5·6, at which pH the

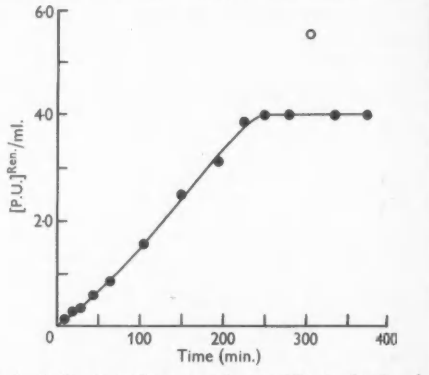

Fig. 4. Activation of parapepsinogen II at pH 4·7 and 35·5°. The activation mixture contained 0·6 ml. of a solution (5 mg./ml.) of parapepsinogen II in 0·02 M-phosphate, pH 6·9, +9·4 ml. of 0·1 M-acetate, pH 4·6. ●, Milk-clotting activity; ○, milk-clotting activity after 5 min. incubation at pH 2·0 and 35·5°.

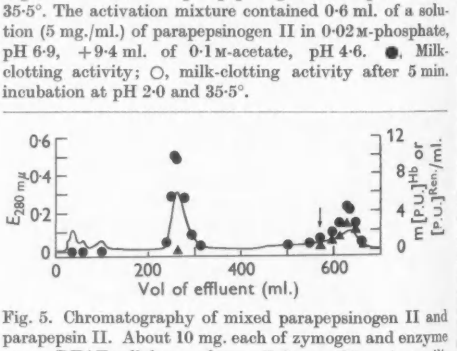

Fig. 5. Chromatography of mixed parapepsinogen II and parapepsin II. About 10 mg. each of zymogen and enzyme on a DEAE-cellulose column (1·2 cm. × 33 cm.) equilibrated with 0·1 M-acetate, pH 5·6. Elution was initially with a gradient obtained by passing 0·4 M-NaCl in 0·1 M-acetate, pH 5·6, into a mixing vessel (140 ml.) containing 0·1 M-acetate, pH 5·6. At the point indicated by the arrow the 0·4 M-NaCl soln. was replaced by M-NaCl in the same buffer. The line represents E at 280 mμ (experimental points omitted); ●, activity against Hb; ▲, milk-clotting activity.

enzyme is stable and the zymogen only very slowly activated. The zymogen appeared first, and when it became clear that the enzyme was emerging as a trailing peak in the gradient to 0·4 M-NaCl, changing the reservoir to M-NaCl in 0·1 M-acetate, pH 5·6, resulted in rapid elution of the enzyme. The recovery of activity of the zymogen was 92 % and of the enzyme 90 %.

DISCUSSION

The results reported above answer one of the questions posed by the similarity of pepsin and parapepsin II: that of the origin of the new enzyme. The enzyme is derived from a zymogen, which in its similarity to pepsinogen reflects the similarity of the enzymes. There seems little doubt that the material obtained here is in fact the zymogen of parapepsin II, since at low pH it shows the same feature of activity against haemoglobin unaccompanied by activity against acetylphenylalanyl-di-iodotyrosine, and, after standing at pH 2·0, it behaves on chromatography at pH 4·0 in exactly the same way as parapepsin II. It has, moreover, one *N*-terminal amino acid, serine, the same as one of the two *N*-terminal amino acids of parapepsin II (serine and leucine or isoleucine) and its molecular weight (40 500) is of the same order as that found for the enzyme (40 700). Its ratio of milk-clotting to haemoglobin-digesting activity, after activation at pH 2·0, is the same as that of parapepsin II.

When pepsinogen is activated to pepsin the molecular weight of the protein falls from about 42 000 (Herriott, 1938*a*) to 35 500 and among the basic peptides split off is the pepsin inhibitor (Van Vunakis & Herriott, 1956). With parapepsinogen II the change in weight appears to be smaller, and this is supported by the small change in specific activity. Comparison of the behaviour of the zymogen and enzyme on chromatography at pH 5·6 shows that the zymogen is more basic; presumably basic fragments are split off on activation. The experiments on activation at pH 4·1 and 4·7 provide an indication that with parapepsinogen II, as with pepsinogen, an inhibitor may be among the fragments split off. Although the activity produced at pH 4·1 had reached its maximum after 1 hr., treatment of this solution at pH 2·0 for 5 min. caused a 50 % increase in milk-clotting activity; a similar increase appeared with zymogen activated at pH 4·7. This effect could be explained by activation of a second component, not activated at pH 4·1 or 4·7, but the evidence of the chromatography, the ultracentrifuge and end-group analysis all indicated that the protein was homogeneous. It is possible that during the activation an inhibitor is produced, so that the enzyme is only partly active, and that at pH 2·0 the enzyme digests the inhibitor (as pepsin digests its inhibitor) so that on return to pH 4·0 the full activity is demonstrable. This explanation of the phenomenon accords well with the fact that parapepsinogen II is inhibited by the pepsin inhibitor.

There is a difference in the kinetics of activation from the behaviour of pepsinogen, which Herriott (1938*b*) found to be activated by an autocatalytic process at all pH values, although the kinetics were complicated at low pH by the interaction of the pepsin and inhibitor. With parapepsinogen II the activation is first-order at pH 4·1, and at pH 4·7 apparently proceeds by both autocatalytic and first-order mechanisms. It may be that the zymogen is activated by H^+ ions and by parapepsin II, and that at pH 4·7 both mechanisms are important, whereas at pH 4·1 the activation by H^+ ions masks the autocatalytic reaction.

Since parapepsinogen II has been found with pepsinogen in extracts of single mucosae it is clear that the zymogen is not a genetically controlled aberrant form of pepsinogen. This still leaves unanswered the question of the biological function of parapepsin II. As a digestive enzyme it seems that it can do little that pepsin cannot do, since its specificity is slightly narrower than that of pepsin (Ryle & Porter, 1959), and its pH optimum, at least for the later stages of digestion of haemoglobin, is the same as that of pepsin. It is slightly more stable in alkaline solution than pepsin but it is not easy to see how this could be significant. It is perhaps most likely that it has some specific action against a peptide structure, resistant to pepsin, which does not appear in any of the substrates against which it has been tested.

Tang, Wolf, Caputto & Trucco (1959) have isolated a crystalline enzyme, gastricsin, from human gastric juice which has properties not unlike those of parapepsin II (though they have not studied its specificity) and it is possible that their enzyme is the human version of parapepsin II.

No evidence has been found for the presence in extracts of pig fundic mucosa of a zymogen corresponding to parapepsin I or for the presence of parapepsin I itself, which is stable at the pH of the eluting buffer. The recovery of protein from the chromatograms (measured by E at 280 mμ) has usually been close to 100 %, so that large quantities of the enzyme or zymogen cannot have been left on the columns. It is possible that the material is not extracted from the mucosa under the conditions used here, or that it occurs in the mucosa of only a few pigs.

SUMMARY

1. The zymogen of parapepsin II has been isolated from extracts of porcine gastric mucosa.

2. The zymogen is rapidly converted into the

enzyme at pH 2·0 and more slowly at pH values above 4·0.

3. The zymogen has one *N*-terminal residue and a molecular weight close to that of the enzyme.

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REFERENCES

Anson, M. L. (1938). *J. gen. Physiol.* **22**, 79.
Archibald, W. J. (1947). *J. phys. Chem.* **51**, 1204.
Berridge, N. J. (1955). In *Methods in Enzymology*, vol. 2, p. 69. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Blackburn, S. & Lowther, A. G. (1951). *Biochem. J.* **48**, 126.
Charlwood, P. A. (1957). *Trans. Faraday Soc.* **53**, 871.
Herriott, R. M. (1938*a*). *J. gen. Physiol.* **21**, 501.
Herriott, R. M. (1938*b*). *J. gen. Physiol.* **22**, 65.
Herriott, R. M. (1948). In *Crystalline Enzymes*, 2nd ed., p. 259. Ed. by Northrop, J. H., Kunitz, M. & Herriott, R. M. New York: Columbia University Press.
Jacobs, S. (1959). *Nature, Lond.*, **183**, 262.
Levy, A. L. (1954). *Nature, Lond.*, **174**, 126.
Moore, S. & Stein, W. H. (1954). *J. biol. Chem.* **211**, 907.
Northrop, J. H., Kunitz, M. & Herriott, R. M. (1948). *Crystalline Enzymes*, 2nd ed., p. 305. Ed. by Northrop, J. H., Kunitz, M. & Herriott, R. M. New York: Columbia University Press.
Peterson, E. A. & Sober, H. A. (1956). *J. Amer. chem. Soc.* **78**, 751.
Porter, R. R. (1957). In *Methods in Enzymology*, vol. 4, p. 221. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Ryle, A. P. & Porter, R. R. (1959). *Biochem. J.* **73**, 75.
Tang, J., Wolf, S., Caputto, R. & Trucco, R. E. (1959). *J. biol. Chem.* **234**, 1174.
Van Vunakis, H. & Herriott, R. M. (1956). *Biochim. biophys. Acta*, **22**, 537.

Biochem. J. (1960) **75**, 150

Effect of Ethanol on Respiration of Rat-Brain-Cortex Slices

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Potassium ions and some other agents enhance the respiration and glycolysis of nervous tissue *in vitro* (Ashford & Dixon, 1935; Dickens & Greville, 1935), as do applied electrical pulses (McIlwain, 1951*a*). The respiration of stimulated tissue is affected by narcotics at pharmacologically active concentrations (Bronk & Brink, 1951; McIlwain, 1953*a*; Buchel, 1953), whereas that of unstimulated tissue is not.

Heavy ethanol intoxication in human subjects is associated with diminished cerebral oxygen consumption (Hine, Shick, Margolis, Burbridge & Simon, 1952; Battey, Heyman & Patterson, 1953). Recent work with potassium-stimulated brain-cortex tissue indicates that its oxygen consumption is slightly but significantly depressed at 'physiological' ethanol concentrations (Ghosh & Quastel, 1954; Sutherland, Hine & Burbridge, 1956; Fischer, 1957; Beer & Quastel, 1958*b*), whereas glucose consumption and lactic acid accumulation appear to be enhanced (Sutherland *et al.* 1956). Ethanol in corresponding concentrations slightly elevates unstimulated respiration, at least in the initial phase of the experiment (Fuhrman & Field, 1948; Ghosh & Quastel, 1954; Sutherland *et al.* 1956; Beer & Quastel, 1958*b*).

Here we report studies *in vitro* on the oxygen and glucose consumption and lactic acid formation in stimulated and unstimulated slices of cerebral cortex from the rat. The first aim was to determine whether ethanol had any effect at all in our experimental conditions. A depression of the respiration was observed in tissues stimulated electrically or with potassium chloride. Since changes in glucose concentration did not influence the ethanol effect, it was concluded that there cannot be any competition between ethanol and glucose for access to the enzymes. The nature of the non-competitive inhibition could not be defined.

MATERIAL AND METHODS

Apparatus. All experiments were performed with conventional Warburg constant-volume respiratory manometers and vessels of 13–16 ml. volume. The flasks for electrical stimulation of tissue were modified from the type described by McIlwain (1951*b*) as 'conical electrode vessels E' (Fig. 1). Before experiments, silicone grease was applied to the base of the platinum wires. After each experiment, the electrodes were removed, washed with detergent, rinsed with water, dipped for 15 sec. into dichromate–H_2SO_4 solution, rinsed with water and dried. To facilitate the application and removal of the electrodes, the

vessels were made wide-necked and provided with ground-glass collars for fitting them to the manometers. Calibration was done with mercury: the electrodes were of pure silver and their volume was calculated from their weight.

The stimulators were similar in principle to that described by McIlwain (1951*a*). Condensers (4 μF) operated with direct current from the mains (125 v) were charged and discharged through the vessels by means of autoradio vibrators (Philips AP6014). The current to the condensers was regulated with variable resistors. Vibrator frequency was 90–100 cyc./sec., as stated by the factory. Each vessel was connected with a separate stimulator. Switches were arranged so that voltage and current could be measured at any time during the course of an experiment in each of the stimulated vessels. Voltage was measured at the flasks by means of an electronic voltmeter and current with an ordinary ammeter. A voltage gradient between the electrodes of 1·6–1·8 v/mm. was maintained during stimulation. Equipment was not available for measurements of shape, frequency and time-constant of the pulses, and respiratory response remained the practical criterion of successful stimulation. For the stimulation by K^+ ions 0·1 M-KCl was used.

Experimental procedure. The experimental conditions were as follows: 2·5 ml. of phosphate–glucose medium (McIlwain, 1951*a*) with 6 mM-glucose was used if not otherwise stated; concentration of ethanol was 0·087 M (0·4 %), when used; gas phase was pure oxygen; absorption of carbon dioxide was by 0·1 ml. of KOH (20 %) on folded filter paper; temperature was 37·5°, except when otherwise stated; shaking was at 96 complete strokes/min.; initial gassing and equilibration were for 5 + 10 min. at experimental temperature; after transfer of slices to vessels, they were gassed for 3 min., the required ethanol or KCl solutions (0·2 ml.) were pipetted into side arms and equilibration for 10 min. was allowed; after this, ethanol was tipped into the main compartment and stimulation began; the manometer reading was taken every 10 min. for 1 hr., beginning 5 min. after commencement of stimulation.

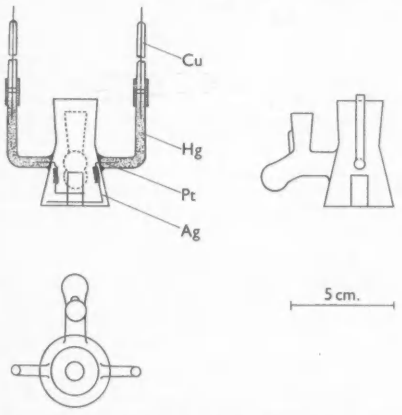

Fig. 1. Vessel employed for application of pulses to tissue slices. Connexions to stimulators are made with platinum lead-in wires (0·5 mm. diam.), side arms are filled with mercury and copper wire in glass tubing is attached to the side arms by means of rubber joints. The concentric electrodes are made of 0·5 mm. silver wire. The centre electrodes ends in a helix pushed on the lead-in wire. The helical connexion of the larger electrode at the bottom is flexibly attached to the ring.

Electrical pulses passed at the chosen voltage through vessels containing experimental medium caused no measurable changes of pressure. Unstimulated tissue respired similarly in electrode flasks and in standard flasks. As a rule, experiments were set up with two standard vessels and two electrode vessels, one of each being used for controls and the other for ethanol exposure.

Adult rats from the Laboratory supply were used, with males and females in equal proportion in each experimental series. Slices were prepared in the cold room with cooled equipment and ice-cold experimental medium for cooling the brain on exposure. Preparation was as described by McIlwain (1951*a*), except that the cortex was not unfolded: instead the hemispheres were placed on the lateromedial surface in a guiding template, and slices (0·35 mm.) were cut directly, three slices being taken from each hemisphere. It was found essential to excise and cool the brain in at most 150 sec. to ensure full respiratory response to both potassium and electrical stimulation. Of the slices from one hemisphere, one (25–40 mg.) was used for determination of respiration of unstimulated tissue and two slices (50–70 mg.) for determination of respiration of stimulated tissue. To avoid systematic errors in the procedure, the following alternations were made: the first and second or the second and third slices were used for stimulation; tissue from the left or the right hemisphere was used as control, that from the other was exposed to ethanol; the first or the second pair of vessels contained control medium, the other ethanol–medium. The dry weight of twelve slices prepared in the same way as those used for respiration experiments was determined by drying for 4 hr. at 110° and was 20·7 % (S.D. 0·37 %).

Usually 10–15 min. elapsed from decapitation of the animal until the vessels were in the thermostat. After the last manometer reading had been taken, samples (1 ml.) were rapidly withdrawn for determination of glucose (Somogyi & Nelson, 1945) and for lactic acid determination (Barker & Summerson, 1941). In our hands, these analytical methods both gave S.D. 4 %.

Comparison was made of respiration during the first hour after equilibration of tissue slices stimulated during the whole period with that of unstimulated slices during the same period. This was because a continuous fall in response to stimulation was found in our experiments, as in those of Sutherland *et al.* (1956). Calculations of the results were based on unit fresh weight. No corrections were made for lactic acid present or glucose consumed at the beginning of the reading of the manometers, since such correction would not reduce the variation.

The statistical significance of the ethanol effect was calculated by the *t* test for non-independent means, since in every experiment the tissue in the control medium and in the ethanol-containing medium was from the same brain. The effect of other experimental variables was evaluated by Student's *t* test.

RESULTS

Unstimulated tissue

Oxygen consumption. During the experimental period, there was no indication of change in respiratory rate except that maximum uptake of oxygen was apparently not attained at the commencement of readings (Fig. 2). Changing glucose concentration did not influence oxygen consumption (see also Table 2). Ethanol caused an increase in respiration. This was statistically significant in the series with 6 mM-glucose as well as for the combined results for the experiments with all three glucose concentrations.

Glucose consumption. Glucose consumption was approximately the same at 3 and 6 mM concentration in the medium (Table 1). There was a significant increase when the initial glucose concentration was 10 mM (Table 2). Ethanol caused a significant augmentation of glucose consumption, both for the series with 6 mM-glucose and for the combined results for all three glucose concentrations. The change induced by ethanol was of the same order of magnitude and in the same direction as that in oxygen consumption.

Lactic acid formation. The formation of lactic acid (Table 1) was not significantly influenced by change in initial glucose concentration of the medium or by the presence of ethanol.

Glucose loss in terms of oxidation and glycolysis. Balance calculations were performed assuming that 6 molecules of oxygen are consumed for each molecule of glucose completely oxidized, or 2 molecules of lactic acid formed from each molecule of glucose in glycolysis. The figures given in the last column of Table 1 suggest that glucose was partly metabolized to compounds not measured in the present experiments. McIlwain, Anguiano & Cheshire (1951) performed similar balance calculations and found that all glucose that disappeared could be accounted for by respiration and glycolysis. Their experiments were, however, extended over at

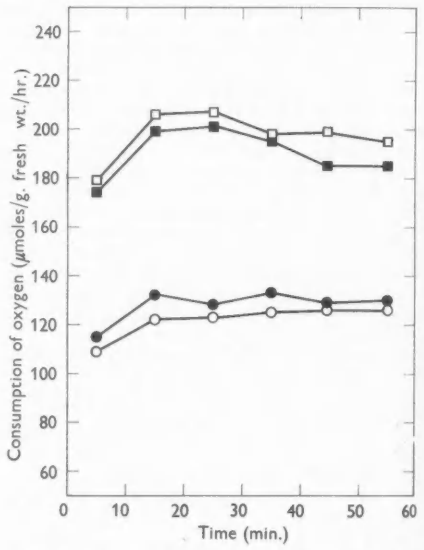

Fig. 2. Time course of respiration of rat-brain-cortex slices incubated *in vitro* in phosphate medium with 6 mM-glucose. ○, Without stimulation and without ethanol (mean of 41 expts.); ●, without stimulation in the presence of 0·087 M-ethanol (41 expts.); □, 0·1 M-KCl without ethanol (23 expts.); ■, 0·1 M-KCl in the presence of 0·087 M-ethanol (21 expts.).

Table 1. *Oxygen consumption, glucose consumption and lactic acid formation of unstimulated cerebral-cortex tissue*

The results are expressed as μmoles/g. of fresh tissue/hr. and standard deviations are given. In the column headed Experimental conditions, control series (C) and ethanol series (E) are indicated. Initial glucose concentration of the medium is given. *n*, Number of experiments. The ethanol concentration was 0·087 M. The level of significance of effects of ethanol is indicated in the footnotes. Figures in the last column show the percentage of glucose loss to be accounted for by the oxygen consumption and lactic acid formation, assuming that 6 molecules of O_2 are consumed or 2 molecules of lactic acid are formed for each molecule of glucose lost.

Experimental conditions	Concn. of glucose (mM)	*n*	Oxygen consumed (μmoles)	Glucose consumed (μmoles)	Lactic acid formed (μmoles)	Loss of glucose accounted for (%)
C	3	13	128±17	78±28	67±18	81·0
E	3	13	132±16	86±32	63±19	62·2
C	6	41	122±17	70±9	75±6	81·8
E	6	41	132±15*	86±10†	75±6	75·1
C	10	9	126±11	102±51	77±31	58·3
E	10	9	127±13	110±47	80±20	55·6

* $P < 0·01$ (combined ethanol effect at all concentrations of glucose $P < 0·01$).
† $P < 0·001$ (combined ethanol effect at all concentrations of glucose $P < 0·001$).

Table 2. *Evaluation of the effect of changing glucose concentration and temperature on respiration and metabolism of unstimulated cerebral-cortex tissue*

The control series with 6 mM-glucose at 37·5° serves as a standard of reference. Significance of differences between means has been obtained by *t* tests and is indicated as the *P* value. NS, Not significant.

Variable	3 mM	10 mM	30°	41·2°
Oxygen consumed	NS	NS	<0·001	<0·001
Glucose consumed	NS	<0·001	<0·05	<0·005
Lactic acid formed	NS	NS	NS	<0·025

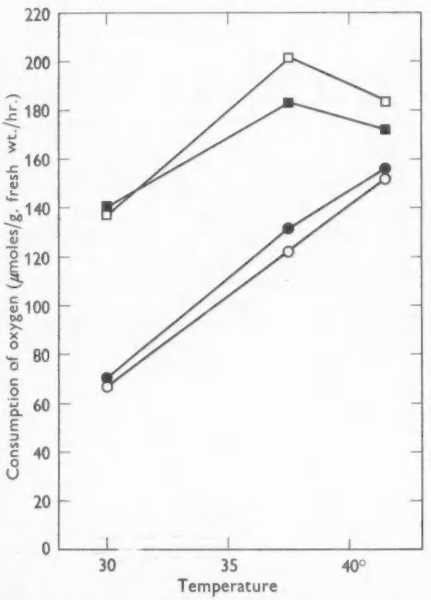

Fig. 3. Effect of temperature on the oxygen consumption of rat-brain-cortex slices incubated in phosphate medium with 6 mM-glucose. ○, Unstimulated, without ethanol (mean of 10 expts. at 30 and 41·5° and 41 expts. at 37·5°); ●, unstimulated in the presence of 0·087 M-ethanol (no. of expts. as without ethanol); □, stimulated without ethanol (10 expts. at 30 and 41·5°; 20 at 37·5°); ■, stimulated in the presence of 0·087 M-ethanol (no. of experiments as without ethanol).

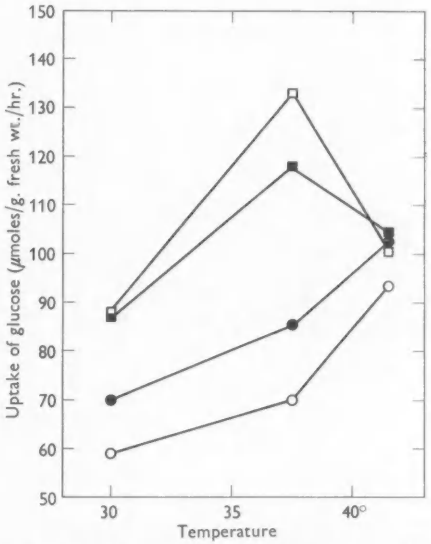

Fig. 4. Effect of temperature on the glucose uptake of rat-brain-cortex slices incubated in phosphate medium with 6 mM-glucose. ○, Unstimulated, without ethanol (mean of 10 expts. at 30° and 41·5° and 41 expts. at 37·5°); ●, unstimulated in the presence of 0·087 M-ethanol (no. of expts. as without ethanol); □, stimulated without ethanol (10 expts. at 30 and 41·5°; 20 at 37·5°); ■, stimulated in the presence of 0·087 M-ethanol (no. of expts. as without ethanol).

least 150 min. We found that in seven experiments of 2 hr. duration, glucose consumption was 51 ± 8 μmoles/g. of fresh tissue/hr. Lactic acid formation was 69 ± 15 μmoles/g. of fresh tissue/hr. Oxygen consumed and lactic acid formed corresponded to 106% of the glucose loss.

Effect of temperature. Within the temperature range covered in our experiments, unstimulated respiration showed a linear relationship to temperature with Q_{10} 2·1, i.e. typical of biological phenomena dependent on enzymic reactions (Fig. 3). Glucose consumption was also significantly influenced by temperature (Fig. 4 and Table 2). A drop in temperature did not alter lactic acid production, whereas this was significantly increased by a rise in temperature to +41·5° (Fig. 5). Within the limits of experimental variation, the ethanol effect appeared to be unaltered by change of temperature except in the lactic acid production.

Stimulated tissue

Oxygen consumption. The values are given in Table 3. Evaluation of the results was based on the absolute values. This was because Fischer (1957) and Beer & Quastel (1958*b*) noticed that

with the same concentration of ethanol oxygen consumption of stimulated tissue could be depressed to lower values than that of unstimulated tissue. Potassium and electrical stimulation both increased the oxygen consumption to about 65% above the control unstimulated value at 37·5° with 6 mM-glucose. Variation in glucose concentration did not influence oxygen consumption significantly (Table 4). The respiration rate of electrically stimulated tissue plotted against time showed a progressive fall. The slopes were identical at all glucose concentrations (Fig. 6) and differed markedly from that of the respiration during potassium stimulation (Fig. 2).

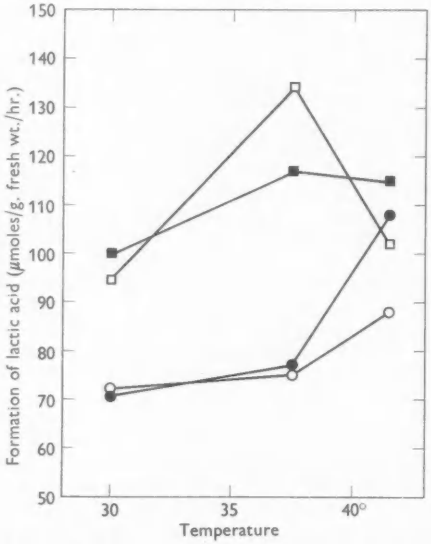

Fig. 5. Effect of temperature on the lactic acid formation of rat-brain-cortex slices incubated in phosphate medium with 6 mM-glucose. ○, Unstimulated without ethanol (mean of 10 expts. at 30° and 41·5° and 41 expts. at 37·5°); ●, unstimulated in the presence of 0·087 M-ethanol (no. of expts. as without ethanol); □, stimulated without ethanol (10 expts. at 30° and 41·5°, 20 at 37·5°); ■, stimulated in the presence of 0·087 M-ethanol (no. of expts. as without ethanol).

Ethanol had a statistically significant inhibitory effect. Potassium-stimulated tissue was apparently more resistant than electrically stimulated tissue. The inhibition caused by ethanol was the same at all glucose concentrations employed. This result was confirmed by statistical treatment and a Lineweaver–Burk plot based on the average oxygen consumption during the experimental hour. It was further corroborated by the fact that the effect of ethanol did not change with time and hence decreasing glucose concentration.

Glucose consumption. Both methods of stimulation increased the glucose consumption (Table 3). With potassium chloride, glucose consumption was significantly higher than with electrical excitation (Table 4). Increased glucose concentration was paralleled by augmented consumption (Tables 3 and 4). Ethanol decreased the glucose consumption in electrically stimulated tissue, a change in the same direction as in oxygen consumption, whereas there was an increase with potassium-stimulated tissue.

Lactic acid formation. As shown in Tables 3 and 4, potassium stimulation caused a substantially larger increase in lactic acid formation than electrical stimulation. When initial glucose concentration was decreased to 3 mM, lactic acid formation was diminished, whereas there was no effect of change from 6 to 10 mM (Table 3). The effect of ethanol was more irregular than on glucose con-

Table 3. *Oxygen consumption, glucose consumption and lactic acid formation of stimulated cerebral-cortex tissue*

Results are expressed as μmoles/g. of fresh tissue/hr. and standard deviations are given. *P* values for statistically significant differences between control and ethanol series are indicated. In the column headed Experimental conditions, K, potassium stimulation; S, electrical stimulation; C, control; E, ethanol (0·087 M). Initial glucose concentration is given. *n*, Number of experiments. NS, not significant.

Experimental conditions	Concn. of glucose (mM)	*n*	Oxygen consumed (μmoles)	*P* <	Glucose consumed (μmoles)	*P* <	Lactic acid formed (μmoles)	*P* <	Loss of glucose accounted for (%)
K, C	6	23	204±13	0·025	171±38	0·025	190±30	NS	75·4
K, E	6	21	196±13		189±45		195±30		68·9
S, C	3	13	192±19	0·025	91±21	NS	63±15	NS	69·8
S, E	3	13	175±17		81±17		69±19		78·6
S, C	6	20	202±13	0·001	133±27	0·001	134±21	0·001	75·7
S, E	6	20	183±15		118±26		117±26		75·4
S, C	10	9	200±11	0·025	168±39	NS	128±25	NS	57·9
S, E	10	9	184±18		145±47		125±39		64·3

Table 4. *Evaluation of the effect of changing experimental conditions on respiration and metabolism of stimulated cerebral-cortex tissue*

The electrically stimulated control series with 6 mM-glucose at 37·5° serves as standard of reference. Significance of difference between means has been obtained by *t* tests and is indicated as the *P* value. NS, Not significant.

Variable	0·1 M-KCl	3 mM	10 mM	30°	41·5°
Oxygen consumed	NS	NS	NS	<0·001	NS
Glucose consumed	<0·001	<0·001	<0·025	<0·001	<0·001
Lactic acid formed	<0·001	<0·001	NS	<0·01	<0·001

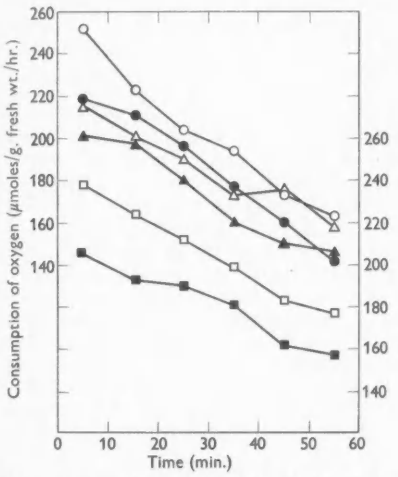

Fig. 6. Time course of respiration of electrically stimulated rat-brain-cortex slices incubated at 37·5° in phosphate medium with different initial glucose concentration. △, 3 mM-Glucose, no ethanol (mean of 13 expts.); ▲, 3 mM-glucose, 0·087 M-ethanol (13 expts.); □, 6 mM-glucose, no ethanol (20 expts.); ■, 6 mM-glucose, 0·087 M-ethanol, (20 expts.); ○, 10 mM-glucose, no ethanol (9 expts.); ●, 10 mM-glucose, 0·087 M-ethanol (9 expts.). Left ordinate, rate of oxygen uptake with 3 and 10 mM-glucose; right ordinate, rate with 6 mM.

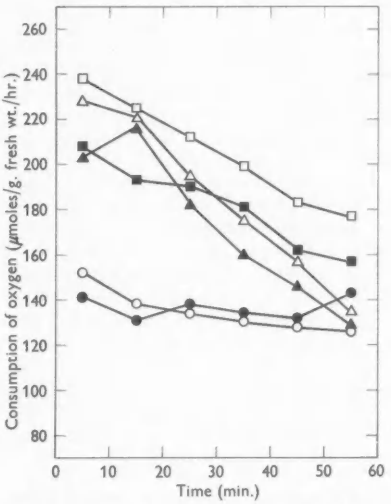

Fig. 7. Time course of respiration of electrically stimulated rat-brain-cortex slices incubated in phosphate medium with 6 mM-glucose at different temperatures. ○, At 30°, no ethanol (mean of 10 expts.); ●, at 30°, 0·087 M-ethanol (10 expts.); □, at 37·5°, no ethanol (20 expts.); ■, at 37·5°, 0·087 M-ethanol (20 expts.); △, at 41·5°, no ethanol (10 expts.); ▲, at 41·5°, 0·087 M-ethanol (10 expts.).

sumption. In the largest series with electrical excitation, however, there was a clear drop in lactic acid formation. This was also statistically significant ($P < 0·005$) when calculated for the combined results from the experiments with all three glucose concentrations at 37·5°.

Glucose loss in terms of oxidation and glycolysis. Results of balance calculations performed as for unstimulated tissue are shown in Table 3 (last column). The same remarks apply as for the unstimulated tissue. In seven experiments of 2 hr. duration, glucose consumption was 64 ± 7 μmoles/g. of fresh tissue/hr. Lactic acid formation was 79 ± 15 μmoles/g. of fresh tissue/hr. Oxygen consumed and lactic acid formed corresponded to 99·5 % of the glucose loss.

Effect of temperature. Both at 30° and 41·5° oxygen consumption of the electrically excited tissue was lower than at 37·5° (Fig. 3). The time course was strongly influenced by temperature (Fig. 7). The slopes of the curves representing rate of oxygen consumption as a function of time become steeper with increase in temperature. The average stimulation during the experimental hour was 104 % above the control at 30° (falling from 128 % during the first 10 min. to 90 % during the last period), 65 % at 37·5° (falling from 95 to 45 %), and 21 % at 41·5° (falling from 50 to −12 %). Because of this, the Q_{10} was calculated for the first 10-min. period of manometer reading on the basis of the results obtained at 30° and 37·5°. It was 1·75, i.e. considerably lower than for the

unstimulated tissue. Change in temperature caused highly significant changes in glucose consumption (Fig. 4 and Table 4). The inhibitory effect of ethanol on oxygen consumption had completely disappeared at 30° (Figs. 3 and 7). At 41·5° it was approximately the same as at 37·5°.

DISCUSSION

Stimulation of brain tissue *in vitro* appears to be possible only with preparations in which the cell structure is largely intact (Kimura, 1937). Under certain conditions applied electrical pulses alter the metabolism of homogenates and mitochondrial preparations (Abood, Gerard & Ochs, 1952), but these alterations differ qualitatively and quantitatively from those obtained with slices (Narayanaswami & McIlwain, 1954). Induced electrical activity of the type obtainable in undercut isolated slabs of cortical tissue (Burns, 1958) has not been observed in brain-cortex slices as used in manometric experiments (McIlwain & Ochs, 1952). However, there are other important metabolic and physiologic parallels between electrically stimulated brain slices and the brain *in vivo* (cf. Forda & McIlwain, 1953; McIlwain, 1954), whereas unstimulated slices represent conditions more remote from those prevailing in living, intact brain. Because of this, our results with stimulated and unstimulated tissue will be discussed separately.

Unstimulated tissue

Effect of ethanol. Increase in oxygen consumption when ethanol is added in relatively low concentration to media containing glucose as substrate has been observed by other workers (Fuhrman & Field, 1948; Ghosh & Quastel, 1954; Sutherland *et al.* 1956; Beer & Quastel, 1958*b*). The mechanism by which this occurs is obscure. It has been assumed that the elevated respiration is due to oxidation of the ethanol. Several investigators have been unable to show metabolism of ethanol in brain tissue (Bartlett & Barnet, 1949; Masoro, Abramovitch & Birchard, 1953; Beer & Quastel, 1958*a*). On the other hand, Burbridge, Sutherland, Hine & Simon (1959) have reported an appreciable consumption of ethanol in rat-brain slices. In our experimental conditions, oxidation of ethanol by rat-brain slices could not be demonstrated, whereas the ethanol was normally metabolized by liver slices. Ethanol has not been observed to act as an uncoupling agent of the oxidative phosphorylation (Truitt, Bell & Krantz, 1956). Our results demonstrate that the increase in respiration is accompanied by increased glucose consumption. Thus glucose probably furnishes the substrate for the enhanced oxidation. Low concentrations of ethanol slightly depolarize the neuronal membrane and increase neuronal excitability (Wright, 1947; Gallego, 1948; Larrabee & Posternak, 1952), and thus functional activation may be the underlying cause of the shift of respiration to a higher level.

Glucose concentration. The change in concentration of glucose employed did not influence the rate of respiration. This is in agreement with McIlwain's (1953*b*) observation that unstimulated tissue will be affected only when the glucose concentration drops to the range 0·5–1·5 mM.

Temperature. In a study on the respiration of unstimulated rat-brain tissue in the temperature range 0·2–47·5°, Field, Fuhrman & Martin (1944) demonstrated a non-linear relationship between Q_{O_2} and temperature, Q_{10} 2·128 over the range 10·0–37·5° and a progressive decrease in respiration with time when the temperature was over 40°. Field *et al.* made a larger number of observations than we did in the temperature range 30·0–41·5°, and their experiments were extended over periods of at least 90 min. This may explain why our results suggest a simple linear relationship of oxygen consumption to temperature, which is probably not a correct picture of the necessarily complicated responses. Our short experimental period makes it understandable why there is no significant drop in the rate of oxygen uptake towards the end of our experiments at 41·5°. Glucose uptake and lactic acid formation are higher at 41·5° than a linear dependence would imply.

Stimulated tissue

Effect of ethanol. The respiration and consumption of glucose were clearly decreased when ethanol was present. The opposite effects of ethanol on stimulated and on unstimulated tissue is an apparent paradox. However, if our suggestion is correct, that the depolarizing action of ethanol is related to its effect on respiration of unstimulated tissue, then these seemingly contradictory phenomena may both be related to the function of excitable neuronal structures. The ethanol concentration in our experiments represents approximately half the lethal blood concentration for rats. The magnitude of the depression of respiration obtained with potassium-stimulated tissue corresponds well to that observed by other investigators (Ghosh & Quastel, 1954; Sutherland *et al.* 1956; Beer & Quastel, 1958*b*). When condenser pulses were used for stimulation, more pronounced inhibition was obtained.

Comparison of ethanol and other depressants. In the presence of depressants, potassium stimulation and electrical stimulation have a different effect on glucose consumption and lactic acid accumulation. Sutherland *et al.* (1956) observed that ethanol (0·056 M) induced a significant increase in glucose uptake and lactic acid formation of tissue stimu-

lated with potassium. In our experiments with potassium stimulation, both were slightly larger with ethanol (0·087 M) than without. McIlwain (1953*a*) found that depressants caused a fall in respiratory rate, glucose consumption and lactic acid accumulation in stimulated isolated brain tissue, but that lactic acid formation was much less affected in tissue stimulated with potassium (0·033 M) than in electrically excited tissue. Thus our results with electrically stimulated tissue in the presence of ethanol show agreement with those obtained by McIlwain using other depressants. They also indicate that ethanol acts without delay and, during a 1 hr. period, independently of time. The same conclusion has been drawn with respect to ethanol by Beer & Quastel (1958*b*) and with respect to other depressants by McIlwain (1953*a*).

Effect of temperature. As is demonstrated by the result obtained at 41·5°, the respiratory and metabolic response to rising temperature will be an increase up to some critical temperature, above which a drop will occur. Progressive warming above body temperature also depresses the functional activity of the spinal cord (Brooks, Koizumi & Malcolm, 1955). It should further be noted that the slope of the graphs expressing rate of respiration as a function of time becomes steeper with increasing temperature (Fig. 7). This may in part be attributable to progressive inactivation of enzyme systems or cellular elements, which tends to be more rapid at higher temperatures. The relative increase in respiration when pulses are applied is higher at 30° than at 37·5°. This may be related to the known fact that whereas at moderately lowered temperature the excitation threshold of neuronal elements in the spinal cord is increased, the amplitude and duration of action potentials is also markedly larger (for review and experimental evidence, cf. Brooks *et al.* 1955).

Mechanism of inhibitory effect of ethanol. The mechanism of ethanol action is not known. The respiratory response of brain tissue to stimulation appears to be dependent on preservation of cell structure, on the operation of the citric acid cycle and on the presence of glucose or pyruvate as substrate (Kimura, 1937; McIlwain & Gore, 1953; Kratzing, 1953; McIlwain, 1953*b*, *c*; Beer & Quastel 1958*b*). Because pharmacologically active concentrations of ethanol depress the respiration of stimulated tissue only, it seems evident that the effect of ethanol is directed towards either the functional response to stimulation or the metabolic system supporting this response.

Himwich (1956) has suggested that ethanol may interfere with the metabolism of energy-rich phosphates. As mentioned in the preceding section, ethanol does not appear to act by uncoupling of oxidative phosphorylation. The hypothesis that conversion of ethanol into acetaldehyde contributes to its action in brain slices has been tentatively supported (Sutherland *et al.* 1956) but seems untenable in view of the results reported by Beer & Quastel (1958*a*, *b*).

Ethanol does not affect mitochondrial respiration in pharmacologically active concentration (Beer & Quastel, 1958*b*). This observation seems very important in view of the fact that the tricarboxylic acid cycle, fatty acid oxidation, oxidative phosphorylation and terminal-electron transport are all connected with the functioning of structurally organized enzyme systems within the mitochondria (for review, see Green, 1956–57). Results obtained by one of us (Wallgren, 1960) have shown that ethanol and malonate have no additive effect. Thus ethanol does not seem to interfere with any enzyme involved in the dehydrogenation of succinic acid.

Sutherland *et al.* (1956), as well as Ghosh & Quastel (1954), interpret their results as indicating an effect of ethanol on a potassium-sensitive phase in nerve respiration which is concerned with glucose or pyruvate metabolism. Our demonstration that ethanol acts independently of glucose concentration in the medium suggests that its depression of respiration is not due to competition with glucose. Quastel (1952) has shown that some other narcotics non-competitively inhibit pyruvate- or lactate-supported respiration of brain-cortex slices. The available evidence is not at variance with the conclusion of Beer & Quastel (1958*b*) that the membrane of excitable cells is the most likely site for the pharmacological action of ethanol. It also makes it probable that the depression of respiration is a secondary effect due to disturbance of an energy-demanding functional process.

SUMMARY

1. The effect of ethanol on the respiration of unstimulated and stimulated rat-brain-cortex slices was investigated. The consumption of glucose and the formation of lactic acid were also measured.

2. In unstimulated slices at 37·5° the addition of ethanol (0·087 M) increased the consumption of both oxygen (+8 %, $P < 0·01$) and glucose (+23 %, $P < 0·001$), but did not affect the formation of lactic acid.

3. In electrically stimulated tissue at 37·5° ethanol caused a decrease in the respiration (−9·5 %, $P < 0·001$), glucose consumption (−11 %, $P < 0·001$) and lactic acid accumulation (−12·5 %, $P < 0·001$). These changes were unaffected by varying the initial glucose concentration in the medium from 3 to 10 mM. In potassium-stimulated slices the effect of ethanol on the respiration was

less marked and the glucose uptake and lactic acid accumulation increased.

4. In unstimulated slices respiration, glucose consumption and lactic acid formation increased with rise of temperature (30·0–41·5°). In electrically stimulated preparations the functions mentioned above increased from 30° to 37·5° but decreased at 41·5°. Ethanol had no effect at 30°.

5. The relationship of these results to those obtained in earlier investigations with ethanol and other depressants is discussed. It is concluded that the action of ethanol is non-competitive and directed towards some process linked with the physiological functioning of nerve tissue.

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REFERENCES

Abood, L. G., Gerard, R. W. & Ochs, S. (1952). *Amer. J. Physiol.* **171**, 134.
Ashford, C. A. & Dixon, K. C. (1935). *Biochem. J.* **29**, 157.
Barker, S. B. & Summerson, W. H. (1941). *J. biol. Chem.* **138**, 535.
Bartlett, G. R. & Barnet, H. N. (1949). *Quart. J. Stud. Alc.* **10**, 381.
Battey, L. L., Heyman, A. & Patterson, J. L. (1953). *J. Amer. med. Ass.* **152**, 6.
Beer, C. T. & Quastel, J. H. (1958*a*). *Canad. J. Biochem. Physiol.* **36**, 531.
Beer, C. T. & Quastel, J. H. (1958*b*). *Canad. J. Biochem. Physiol.* **36**, 543.
Bronk, D. W. & Brink, F. (1951). *Fed. Proc.* **10**, 19.
Brooks, C. McC., Koizumi, K. & Malcolm, J. L. (1955). *J. Neurophysiol.* **18**, 205.
Buchel, L. (1953). *Anésth. Analg.* **10**, 1.
Burbridge, T. N., Sutherland, V. C., Hine, C. H. & Simon, A. (1959). *J. Pharmacol.* **126**, 70.
Burns, B. D. (1958). *The Mammalian Cerebral Cortex.* London: Edward Arnold Ltd.
Dickens, F. & Greville, G. B. (1935). *Biochem. J.* **29**, 1468
Field, J., Fuhrman, F. A. & Martin, A. W. (1944). *J. Neurophysiol.* **7**, 117.
Fischer, E. (1957). *A.A.A.S. Publ.; Alcoholism*, no. 47 p. 19.
Forda, O. & McIlwain, H. (1953). *Brit. J. Pharmacol.* **8**, 225.
Fuhrman, F. A. & Field, J. (1948). *Proc. exp. Biol., N.Y.*, **69**, 331.
Gallego, A. (1948). *J. cell. comp. Physiol.* **31**, 97.
Ghosh, J. J. & Quastel, J. H. (1954). *Nature, Lond.*, **174**, 28.
Green, D. E. (1956–57). *Harvey Lect.* **52**, 177.
Himwich, H. E. (1956). In *Alcoholism*, p. 291. Ed. by Thompson, G. E. Springfield, Ill.: Charles C. Thomas.
Hine, C. H., Shick, A. F., Margolis, L., Burbridge, T. N. & Simon, A. (1952). *J. Pharmacol.* **106**, 253.
Kimura, Y. (1937). *Sci. Pap. Inst. phys. chem. Res., Tokyo*, **33**, 231.
Kratzing, C. C. (1953). *Biochem. J.* **54**, 312.
Larrabee, M. G. & Posternak, J. M. (1952). *J. Neurophysiol.* **15**, 91.
Masoro, E. J., Abramovitch, H. & Birchard, J. R. (1953). *Amer. J. Physiol.* **173**, 37.
McIlwain, H. (1951*a*). *Biochem. J.* **49**, 382.
McIlwain, H. (1951*b*). *Biochem. J.* **50**, 132.
McIlwain, H. (1953*a*). *Biochem. J.* **53**, 403.
McIlwain, H. (1953*b*). *Biochem. J.* **55**, 618.
McIlwain, H. (1953*c*). *J. Neurol. Psychiat.* **16**, 257.
McIlwain, H. (1954). *Electroenceph. clin. Neurophysiol.* **6**, 93.
McIlwain, H., Anguiano, G. & Cheshire, J. D. (1951). *Biochem. J.* **50**, 12.
McIlwain, H. & Gore, M. B. R. (1953). *Biochem. J.* **54**, 305.
McIlwain, H. & Ochs, S. (1952). *Amer. J. Physiol.* **171**, 128.
Narayanaswami, A. & McIlwain, H. (1954). *Biochem. J.* **57**, 663.
Quastel, J. H. (1952). *Curr. Res. Anesth.* **31**, 151.
Somogyi, M. & Nelson, N. (1945). *J. biol. Chem.* **160**, 69.
Sutherland, V. C., Hine, C. H. & Burbridge, T. N. (1956). *J. Pharmacol.* **116**, 469.
Truitt, E. B., Bell, F. K. & Krantz, J. K. (1956). *Quart. J. Stud. Alc.* **17**, 594.
Wallgren, H. (1960). *Acta physiol. scand.* (in the Press).
Wright, E. B. (1947). *Amer. J. Physiol.* **148**, 174.

Biochem. J. (1960) **75**, 158

Purification of the Two Components of Leucocidin from *Staphylococcus aureus*

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Staphylococcal leucocidin inhibits the respiration (Neisser & Wechsberg, 1901) and produces characteristic morphological changes (Gladstone & van Heyningen, 1957) in the white blood cells of humans and rabbits. It has been shown that this activity results from the synergistic action of at least two substances, since the activity is lost when leucocidin preparations are separated on carboxy-

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methylcellulose into two fractions but is regained when the fractions are mixed (Woodin, 1959). According to their rate of migration on carboxymethylcellulose these fractions were called the fast and the slow fraction of the leucocidin preparation. Chromatographically the fast fraction behaved as a single substance but immunologically several constituents could be distinguished. The electrophoretic behaviour of the fast fraction suggested the formation of complexes, and this may be responsible for the heterogeneous system behaving as a single substance on columns of cation-exchange resins. The slow fraction of leucocidin behaved essentially as a single substance contaminated with some of the constituents of the fast fraction.

The present paper describes the further purification of the fast and slow fractions of the leucocidin preparation. It is now clear that only one of the constituents of each is responsible for the activity and these are referred to below as the fast (F) and slow (S) components of leucocidin. Both components have been crystallized.

MATERIALS AND METHODS

Preparation of culture filtrates. Large-scale production of culture filtrates from the V8 strain of *Staphylococcus aureus* grown in the 'CCY' medium containing a diffusate from Oxoid Yeast extract (Woodin, 1959) was done by Mr R. Elsworth and Mr A. May at the Microbiological Research Establishment, Porton. Ammonium sulphate (750 g.) was stirred into each litre of culture filtrate and the solution left overnight. The bulk of the precipitate rose to the surface and, after the subnatant solution had been siphoned off, was concentrated by centrifuging. The precipitate is called the culture-filtrate slurry.

Fractionation of the culture-filtrate slurry. The 'displacement-eluate 1' and 'displacement-eluate 2' were derived by the method summarized in Fig. 6 of Woodin (1959) with the following modifications.

(*a*) The $(NH_4)_2SO_4$ concentration in the culture-filtrate slurry was reduced to M by adding 0·1 M-phosphate buffer, pH 6·7, at room temperature and the insoluble fraction rejected. With one exception subsequent operations were carried out at 4–7°.

(*b*) The extract in M-$(NH_4)_2SO_4$ was centrifuged at 25 000 rev./min. for 1 hr. and the sediment rejected. The supernatant was diluted to have a conductivity equal to that of 0·1 M-phosphate buffer, pH 6·7, and applied to a column of hydroxylapatite. This was eluted in turn with 0·1 M-phosphate buffer, pH 6·7, 0·14 M-phosphate buffer, pH 6·7, and then with 0·5 M-phosphate buffer, pH 6·7, at room temperature. The last effluent contained the bulk of the two components of leucocidin and was processed on carboxymethylcellulose to give the displacement-eluates 1 and 2 as already described. They were stored in saturated $(NH_4)_2SO_4$. The F and S fractions of the leucocidin preparation were prepared from displacement-eluate 2 as described by Woodin (1959) and stored in saturated $(NH_4)_2SO_4$.

Assay of leucocidin. To enable the measurement of the concentrations of the F and S components of leucocidin the procedure has been modified. For each sample to be assayed dilutions differing by 10% are prepared. An excess (12·5 μg.) of the purified F component (see below) is added to a 1 ml. portion of each dilution and purified S component (2·5 μg.) is added to another 1 ml. portion of each dilution. Antitoxin (0·5 unit) is then added to each tube and the end points are determined by the inhibition of the respiration of macrophages as described previously (Woodin, 1959). The number of test doses (L+) per ml. of the original sample is equal to the dilution at the end point multiplied by the number of units of antitoxin added (i.e. 0·5). The result obtained in the presence of added F component is referred to below as the 'S titre' and that obtained in the presence of added S component as the 'F titre'.

When end points have also been determined by the observation of the morphological changes induced in polymorphonuclear leucocytes (Gladstone & van Heyningen, 1957) the results have not differed.

Staphylococcal antitoxin. This was a pepsin-refined antiserum CPP 76/63 kindly provided by Miss Mollie Barr of the Wellcome Research Laboratories. It was arbitrarily assigned the values 240 units of anti-S component and 240 units of anti-F component.

Assay of deoxyribonuclease. This was done viscometrically by a method similar to that of Laskowski & Seidel (1945). Deoxyribonucleic acid was prepared from calf thymus by the method of Kay, Simmons & Dounce (1952) and a 0·05% solution was prepared in 0·05 M-sodium borate buffer, pH 8·6, containing 0·01 M-$CaCl_2$. This solution had a relative viscosity of 5 at 37° in a viscometer with a flow time of 18 sec. for 1 ml. of water. Preparations with deoxyribonuclease activity were diluted in 0·01 M-$CaCl_2$–0·05 M-sodium borate buffer, pH 8·6, containing 0·2% of gelatin. A portion (0·25 ml.) of the deoxyribonuclease solutions was mixed with 1 ml. of the deoxyribonucleic acid solution at 37° and 1 ml. of the mixture was transferred to the viscometer. Deoxyribonuclease units were calculated by the method of Laskowski & Seidel (1945). For reproducible results it was necessary to dilute the deoxyribonuclease solutions so that they contained less than 6 units/ml.

Chromatographic methods. Dowex-2 (×8) was regenerated and set up in columns by the precedure described by Boman & Westlund (1956), except that after use the top 1 cm. of resin in the column was rejected. The other adsorbants were used in the way described by Woodin (1959).

Protein concentrations. In the partially purified fractions protein concentrations were assessed from the ultraviolet absorption at 280 mμ, assuming $E^{1\%}_{1\,cm.}$ 10. The data given in this paper relating to the properties of the F component and of the S component refer to the concentration calculated from the absorption at 280 mμ and the values $E^{1\%}_{1\,cm.}$ 16·2 and 15·8 for the F and S components respectively.

Nitrogen contents. These were determined by the micro-Kjeldahl method (Markham, 1942; Ma & Zuazaga, 1942). With the purified S component a solution was dialysed against distilled water for several days and dried at 110° in a weighed ampoule; the N content was then determined. With the F component the crystalline material was washed with water till the supernatant gave no precipitate with $AgNO_3$, and dried at 110°. No corrections were made for residual ash content.

Ultracentrifuging. This was done in a Spinco model E analytical ultracentrifuge equipped with the RTIC unit and a phase plate in the schlieren-optical system. Sedimentation coefficients (S) are given in Svedberg units and were calculated from the plot of log x against t, where x is the distance from the centre of rotation to the peak of the gradient curve and t is the time of sedimentation. Apparent diffusion coefficients (D^*) of material sedimenting in the ultracentrifuge were calculated from the equation

$$D=\frac{1}{4\pi t}\left(\frac{A}{H}\right)^2(1-W^2St),$$

where A is the area of the schlieren diagram and H its height, S the sedimentation coefficient in cm./sec./dyne/g., t the time during which diffusion has proceeded and W is the angular velocity. The values of D^* are in cm.^{2}/sec. The concentration of material sedimenting in the ultracentrifuge was calculated from the areas of the gradient curves (corrected for the dilution effect due to the shape of the cell), the phase-plate angle and a calibration constant obtained by forming a boundary between solvent and solution in a synthetic boundary cell at low speed. The partial specific volume ($\bar{V}$) of the F component was calculated from density measurements on a 0·73% solution in 0·2 M-acetate buffer, pH 5·0. The values of $\bar{V}$ are in ml./g.

Boundary electrophoresis. This was done in an Antweiler microelectrophoresis apparatus.

Conductivity measurements. A Mullard conductivity bridge was used for these.

Immunological analysis. This was done by diffusion in agar, by the method of Ouchterlony (1949).

Buffers. Buffers used in the experiments described in this paper had the composition given by Woodin (1959); also 0·01 M-2-amino-2-hydroxymethylpropane-1:3-diol–HCl (tris–HCl) buffer, pH 7·5, prepared by adding HCl to 10 m-moles of tris to give pH 7·5, and diluting to 1 l.

RESULTS

Production of the two components of leucocidin by Staphylococcus aureus

Both the F and the S titres reached maximal values after 8–10 hr., when the culture contained 5–6 g. of organisms/l. The final titres in different batches of culture filtrate varied from 10 L+ of the F component/ml. and 50 L+ of the S component/ml. to 21 L+ of the F component/ml. and 150 L+ of the S component/ml. The latter values correspond to 78 μg. of F component/ml. and 83 μg. of S component/ml.

Recovery of the two components in the preliminary purification

Table 1 lists the yields of the F and S components in the preliminary purification. At all stages the recovery of the S component is less than that of the F component. Little activity could be recovered in the fractions rejected and it is likely that the losses are due to irreversible precipitation.

The extent of separation of the F and S fractions of the leucocidin preparation in the gradient elution from carboxymethylcellulose depends upon the efficiency of the packing of the column, and where significant overlap occurred the overlapping material was processed again.

F component of leucocidin

Purification. The displacement-eluate 1 and the F fraction of the leucocidin preparation were combined. Both were found to contain deoxyribonuclease, some of the properties of which have been described by Privat de Garilhe, Fassina, Pochon & Pillet (1958). They showed that deoxyribonuclease can be adsorbed onto Amberlite CG-50 from a solution in 0·2 M-sodium phosphate buffer, pH 7·0. The F component of leucocidin is not adsorbed under these conditions. However, to achieve separation it is necessary to dialyse the displacement-eluate 1 and the F fraction of the leucocidin preparation against 0·2 M-sodium phosphate buffer, pH 7·0, to remove the $(NH_4)_2SO_4$, and this led to the precipitation of about 30% of the F component in a partly crystalline form. Crystallization at this stage is disadvantageous as the crystalline product contains many impurities.

The method used to purifiy the F component of leucocidin is shown in Fig. 1 and utilizes the finding that leucocidin can be separated from deoxyribonuclease at a lower pH than that used by Privat de Garilhe *et al.* (1958). Dowex-2 (×8) is used to remove pigmented material. After passage through Dowex-2 (×8) the effluent is adjusted to pH 5·0

Table 1. *Recovery of the two components of leucocidin in the preliminary purification*

The culture filtrate processed contained $1·3\times10^6$ L+ of F component and $8·7\times10^6$ L+ of S component.

Stage of purification	F component (% of starting material recovered)	S component (% of starting material recovered)
Fraction soluble in M-$(NH_4)_2SO_4$	54	35
Hydroxylapatite eluate in 0·5 M-phosphate buffer, pH 6·7	31	14
Displacement-eluate 1	19	0·2
Displacement-eluate 2	6	10
F fraction of the leucocidin preparation	4	0·3
S fraction of the leucocidin preparation	2	9

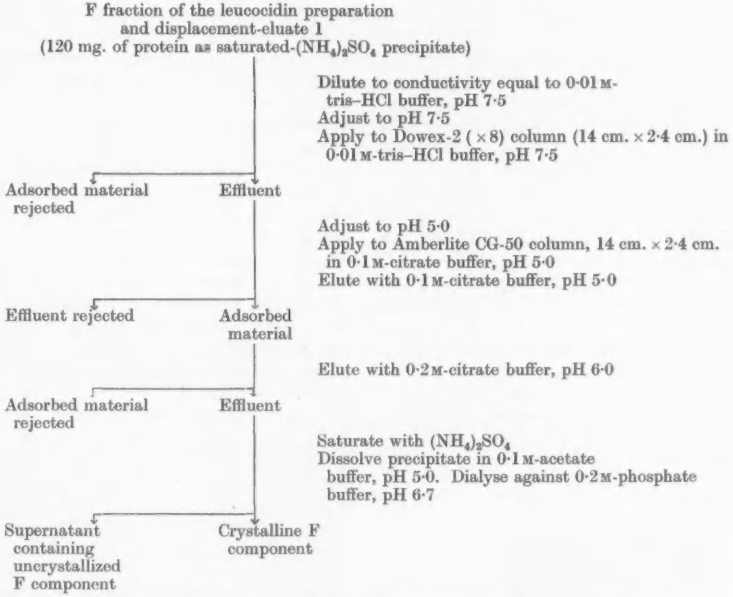
F fraction of the leucocidin preparation and displacement-eluate 1 (120 mg. of protein as saturated-$(NH_4)_2SO_4$ precipitate)
Dilute to conductivity equal to 0·01 M-tris–HCl buffer, pH 7·5
Adjust to pH 7·5
Apply to Dowex-2 (×8) column (14 cm. × 2·4 cm.) in 0·01 M-tris–HCl buffer, pH 7·5
Adsorbed material rejected
Effluent
Adjust to pH 5·0
Apply to Amberlite CG-50 column, 14 cm. × 2·4 cm. in 0·1 M-citrate buffer, pH 5·0
Elute with 0·1 M-citrate buffer, pH 5·0
Effluent rejected
Adsorbed material
Elute with 0·2 M-citrate buffer, pH 6·0
Adsorbed material rejected
Effluent
Saturate with $(NH_4)_2SO_4$
Dissolve precipitate in 0·1 M-acetate buffer, pH 5·0. Dialyse against 0·2 M-phosphate buffer, pH 6·7
Supernatant containing uncrystallized F component
Crystalline F component

Fig. 1. Preparation of the crystalline F component of leucocidin.

Table 2. *Recovery of the F component of leucocidin in the fractionation illustrated in Fig. 1*

Stage in purification	Protein content (mg.)	F component content (L+)	Deoxyribonuclease content (Deoxyribonuclease units)
F fraction of the leucocidin preparation and displacement-eluate 1	120	8700	2×10^7
Effluent from Dowex-2 (×8)	84	8500	$1{\cdot}3 \times 10^7$
Effluent from Amberlite CG-50 in 0·2 M-citrate buffer, pH 6·0	51	7500	(Not measured)
$(NH_4)_2SO_4$-concentrate of the 0·2 M-citrate buffer effluent from Amberlite CG-50	42	5700	3200
Crystalline F component	20	3200	Less than 200

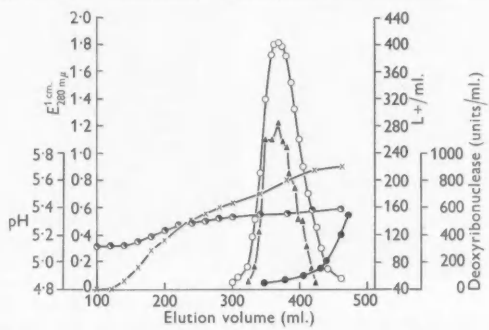

Fig. 2. The elution of the partially purified F component of leucocidin from Amberlite CG-50 by 0·2 M-citrate buffer, pH 6·0. ○, Total protein; ×, pH; ◔, conductivity (arbitrary units); ▲, F component of leucocidin; ●, deoxyribonuclease.

and applied to a column of Amberlite CG-50. The F component is adsorbed and impurities are removed by elution with 0·1 M-citrate buffer, pH 5·0. When the ultraviolet absorption of the effluent has fallen to a constant value and the pH and conductivity of the effluent are those of the 0·1 M-citrate buffer, pH 5·0, the eluent is changed to 0·2 M-citrate buffer, pH 6·0. There is an increase in the conductivity of the effluent to a value approaching that of the inflowing buffer and this is followed by a gradual increase in the pH of the effluent. A single protein peak is eluted above pH 5·4. This stage in the purification is illustrated in Fig. 2. Table 2 gives the recovery of the F component at various stages.

Crystallization. The first 5 % and the last 5 % of the protein peak eluted from Amberlite CG-50 were rejected and the remainder was concentrated and precipitated by dialysis against saturated $(NH_4)_2SO_4$. The precipitate was dissolved in the smallest amount of 0·1 M-acetate buffer, pH 5·0, and the solution was dialysed against 0·2 M-phosphate buffer, pH 6·7. In the course of 2–3 days needle-like crystals or, occasionally, hexagonal-plate-like crystals were deposited (Fig. 3). Recrystallization could be effected by repeating this procedure after solution of the crystals in 0·1 M-acetate buffer, pH 5·0, which requires several hours. About 80 % of the material crystallized in the course of a few days. The crystalline material was stored in 0·2 M-phosphate buffer, pH 6·7, at 4°.

Properties. The N content was 15·5 %; $E^{1\%}_{1\,cm.}$ at 280 mμ was 16·2; the antibody-combining power was 270 L+/mg. Immunological analysis by diffusion in agar showed a single precipitin band (Fig. 4). The supernatant from the first crystallization contained other antigens, indicating the value of the crystallization at this stage in removing trace contaminants.

Ultracentrifuging. All measurements were made at 22°. The twice-crystallized F component, suspended in phosphate buffer, was dissolved in 0·2 M-acetate buffer, pH 5·0, by dialysing a suspension of the crystals against this solvent. A 0·58 % solution was centrifuged at 7000 rev./min. in the synthetic boundary cell and the constant relating the area of the gradient curve to the concentration was calculated. This, together with the optical constants and enlargement factors, gives $1{\cdot}80 \times 10^{-3}$ for the specific refractive increment. The apparent diffusion coefficient ($D^*_{20,w}$) was $8{\cdot}5 \times 10^{-7}$.

A 1·32 % (w/v) solution in 0·2 M-acetate buffer, pH 5·0, was centrifuged at 59780 rev./min. and had

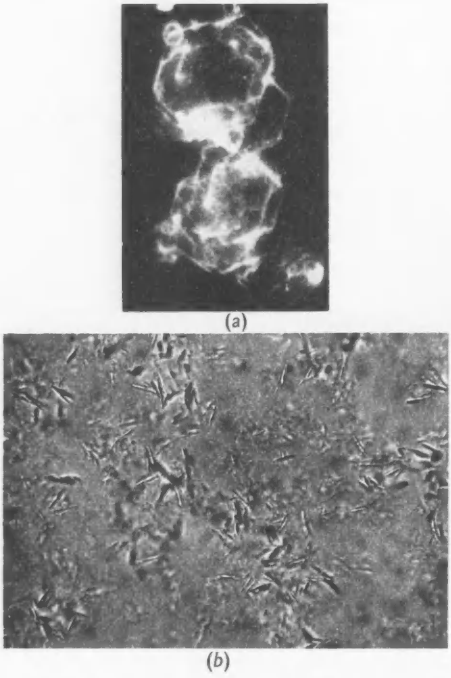

Fig. 3. The two crystalline forms of the F component of leucocidin. (*a*) Hexagonal plates, dark-ground illumination; ×800. (*b*) Needle-like form, light-ground illumination; ×480.

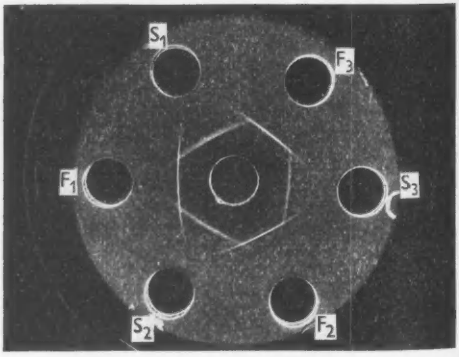

Fig. 4. Immunological analysis of the F and S components of leucocidin. The centre well contained staphylococcal antitoxin CPP 76/63. Recorded after 3 days' diffusion at 37°. F_1, 300 μg. of F component; F_2, 100 μg. of F component; F_3, 33 μg. of F component. S_1, 360 μg. of S component; S_2, 120 μg. of S component; S_3, 40 μg. of S component.

$S_{20,w}$ 3·05. Fig. 5 shows the gradient curve. The concentration of the sedimenting material calculated from the areas of the gradient curves at various times was 95–100 % of the starting material. $D^*_{20,w}$ values were $7{\cdot}14 \times 10^{-7}$–$7{\cdot}42 \times 10^{-7}$, and showed no dependence upon the time of sedimentation. The values S 3·05, D $8{\cdot}5 \times 10^{-7}$, $\bar{V}$ 0·721 give a molecular weight of 32 000.

Electrophoresis of the F component. A 1·5 % (w/v) solution of the crystalline material in 0·1 M-acetate buffer, pH 5·0, showed a single component on boundary electrophoresis. A solution prepared from twice-crystallized material which had been washed with water and freeze-dried retained a slight opalescence after centrifuging at 7000 rev./min. for 20 min. and showed two components on boundary electrophoresis in 0·1 M-acetate buffer, pH 5·0 (Fig. 6). From the appearance of the descending boundary it was clear that the minor, faster component was responsible for the opalescence of the solution. It was removed by centrifuging at 60 000 rev./min. for 30 min. and the supernatant of this centrifuged solution showed a single component on boundary electrophoresis (Fig. 6). A solution prepared from crystalline material which had been stored in the frozen state also showed two components on boundary electrophoresis in 0·1 M-acetate buffer, pH 5·0. It is probable that the minor component is produced from the crystallized F component by denaturation, and freezing or freeze-drying the crystalline material should be avoided.

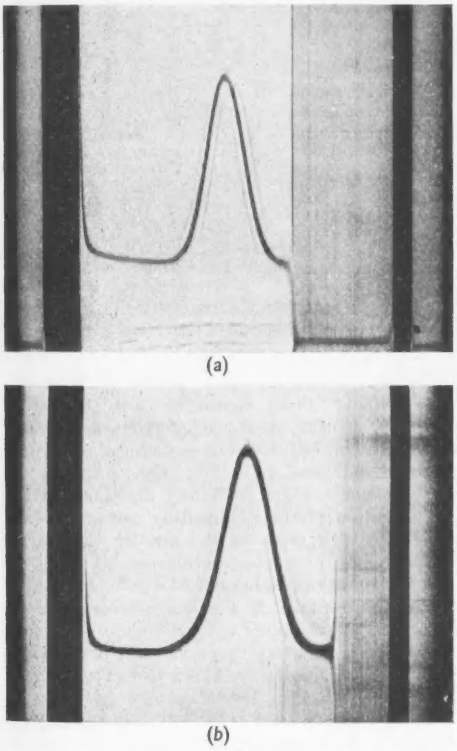

Fig. 5. Schlieren diagrams of the two components of leucocidin sedimenting in the ultracentrifuge. (*a*) F component, 1·32 %, 64 min. after reaching full speed. (*b*) S component, 1·30 %, 85 min. after reaching full speed. In both cases the solvent was 0·2 M-acetate buffer, pH 5·0.

S component of leucocidin

Purification. It was shown that the S fraction of the leucocidin preparation had a single peak on boundary electrophoresis at pH 5·0 and at pH 7·0 (Woodin, 1959). Immunological analysis by diffusion in agar showed a main precipitin band persisting to low antigen concentrations and other precipitin bands which were diluted out at much higher antigen concentrations and appeared to be identical with some of the constituents of the F fraction of the leucocidin preparation. To remove these the S fraction of the leucocidin preparation

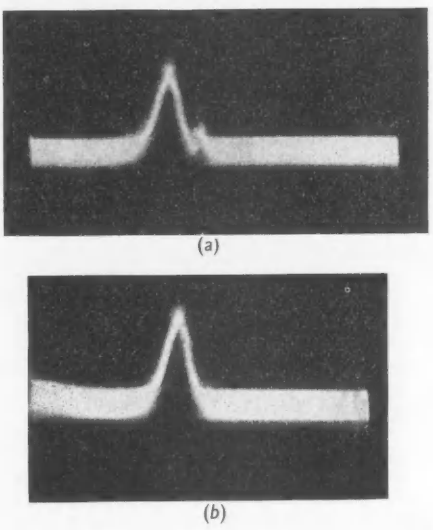

Fig. 6. Boundary electrophoresis of the F component of leucocidin in 0·1 M-acetate buffer, pH 5·0. (*a*) Freeze-dried material before high-speed centrifuging; (*b*) the same material after high-speed centrifuging.

Table 3. *Recovery of the S component of leucocidin on fractionation by the scheme illustrated in Fig.* 1

The analysis of the Amberlite CG-50 effluent is for material remaining after rejection of the first 10% and final 5% of the protein peak.

Stage in the purification	Protein content (mg.)	S component content (L+)	Deoxyribonuclease content (Deoxyribonuclease units)
S fraction of the leucocidin preparation	1000	9×10^5	$5{\cdot}4 \times 10^7$
Effluent from Dowex-2 ($\times 8$)	820	$9{\cdot}5 \times 10^5$	$4{\cdot}5 \times 10^7$
Effluent from Amberlite CG-50 in 0·2M-citrate buffer, pH 6·0	620	$7{\cdot}5 \times 10^5$	$8{\cdot}5 \times 10^3$

was put through the fractionation scheme illustrated in Fig. 1, as far as the elution from Amberlite CG-50 with 0·2M-citrate buffer, pH 6·0, and with the following modification. As the S component was not precipitated by dialysis at pH 7·0, before application of the material to the columns the salt concentrations were adjusted by dialysis and any insoluble material was centrifuged off. Air was excluded from the dialysis sac and the contents were agitated as little as possible, as agitation caused irreversible precipitation of some of the S component in a fibrous form. Fig. 3 of Woodin (1959) indicates a relative displacement of the F and S components on elution from Amberlite CG-50 with a pH gradient. To reduce the contamination of the S component by the F component the first 10% of the protein peak eluted by 0·2M-citrate buffer, pH 6·0, was rejected, and to decrease the deoxyribonuclease content the last 5% of the protein peak was also rejected. The remaining material was dialysed against distilled water and freeze-dried. Table 3 gives the recovery of the S component at various stages. Subsequent to the development of this fractionation scheme the conditions for the crystallization of the S component were discovered and are described below. The properties of the S component have been determined on the freeze-dried material before crystallization.

Properties. The N content was 15·2%, $E^{1\%}_{1\,cm.}$ at 280 mμ was 15·8; the antibody-combining power was 1780 L+/mg. Immunological analysis by diffusion in agar showed a single precipitin band (Fig. 4). The S component has been stored in the freeze-dried form over P_2O_5. The freeze-dried solid is not completely soluble in water, 0·2M-acetate buffer, pH 5·0, or 0·1M-phosphate buffer, pH 6·7, and the amount remaining insoluble appears to increase with storage.

Ultracentrifuging. All measurements were made at 23°. A 0·47% solution of the S component, in 0·2M-acetate buffer, pH 5·0, was centrifuged at 7000 rev./min. and the constant relating the concentration to the area of the gradient curve was determined. This, together with the optical constants and the enlargement factors, gave the value

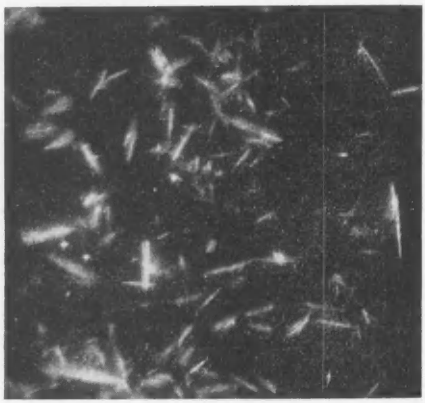

Fig. 7. Crystallized S component of leucocidin. Dark-ground illumination; $\times 600$.

$1{\cdot}87 \times 10^{-3}$ for the specific refractive increment. $D^*_{20,w}$ under these conditions was $7{\cdot}5 \times 10^{-7}$. A 1·30% solution of the S component in 0·2M-acetate buffer, pH 5·0, was centrifuged at 59 780 rev./min. and had $S_{20,w}$ 3·3. Fig. 5 shows the gradient curve, which is slightly asymmetric. The concentration of the sedimenting material calculated from the areas of the gradient curves at various times of sedimentation was 95–101% of that of the starting material. The apparent diffusion coefficients obtained from the gradient curves at different times were $9{\cdot}2 \times 10^{-7}$–$9{\cdot}8 \times 10^{-7}$ and showed no dependence upon the time of sedimentation. The values S 3·3, D $7{\cdot}5 \times 10^{-7}$, $\bar{V}$ 0·721 give a molecular weight of 38 000.

Electrophoresis. A 2·8% solution of the S component in 0·2M-acetate buffer, pH 5·0, showed a single boundary on electrophoresis.

Crystallization. To solutions of the S component, 2% (w/v) in 0·05M-phosphate buffer, pH 6·7, solid $(NH_4)_2SO_4$ was added to give a slightly opalescent solution. Water was added to give a clear solution,

which was left in an open tube at 4–7°. The solution gradually became turbid and had a silky sheen on being stirred. Fig. 7 shows the appearance of the crystalline material suspended in saturated $(NH_4)_2SO_4$.

DISCUSSION

Immunological analysis of the F and S fractions of the leucocidin preparation showed more than one antigen in each fraction (Woodin, 1959). The present work establishes that the leucocidin activity results from synergism between one constituent of each fraction, the F and S components of leucocidin. It is probable that neither component is greatly contaminated with other immunologically active substances or with material of greatly different charge or sedimentation properties. A calculation of the degree of polydispersity has not been attempted as it has been found that both components can be easily denatured and it is likely that some denatured material is present in the most highly purified preparation of each component.

SUMMARY

1. An antibody-combining-power method has been developed for the determination of the concentration of the two components of leucocidin.

2. Both the F and the S fractions of the leucocidin preparation contain deoxyribonuclease.

3. The F and the S components of leucocidin have been purified with Dowex-2 (×8) and Amberlite CG-50.

4. The F component of leucocidin crystallizes from 0·2 M-phosphate buffer, pH 6·7, in the form of needles or as hexagonal plates.

5. The slow component of leucocidin has been crystallized by salting out with ammonium sulphate from 0·05 M-phosphate buffer, pH 6·7, in the form of very fine needles.

6. Both the F and S components of leucocidin behave immunologically as single substances and on electrophoresis or ultracentrifuging show a single boundary.

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REFERENCES

Boman, H. G. & Westlund, L. E. (1956). *Arch. Biochem. Biophys.* **64**, 217.

Gladstone, G. P. & van Heyningen, W. E. (1957). *Brit. J. exp. Path.* **38**, 123.

Kay, R. M., Simmons, N. S. & Dounce, A. L. (1952). *J. Amer. chem. Soc.* **74**, 465.

Laskowski, M. & Seidel, M. K. (1945). *Arch. Biochem.* **7**, 465.

Ma, T. S. & Zuazaga, G. (1942). *Industr. Engng Chem. (Anal.)*, **14**, 280.

Markham, R. (1942). *Biochem. J.* **36**, 790.

Neisser, M. & Wechsberg, F. (1901). *Z. Hyg. InfektKr.* **36**, 299.

Ouchterlony, Ö. (1949). *Acta path. microbiol. scand.* **26**, 507.

Privat de Garilhe, M., Fassina, G., Pochon, F. & Pillet, J. (1958). *Bull. Soc. Chim. biol., Paris*, **40**, 1905.

Woodin, A. M. (1959). *Biochem. J.* **73**, 225.

Biochem. J. (1960) **75**, 165

Comparative Detoxication

7. THE METABOLISM OF CHLOROBENZENE IN LOCUSTS: EXCRETION OF UNCHANGED CHLOROBENZENE AND CYSTEINE CONJUGATES*

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Chlorinated cyclic hydrocarbons are among the most widely used insecticides but sometimes their value is reduced if the insects can metabolize the poison to inactive products. The importance of detoxication mechanisms in strains of some insects resistant to 2:2-bis-(*p*-chlorophenyl)-1:1:1-trichloroethane (DDT) is now well known (cf. Metcalf, 1955), and the natural resistance of the grasshopper family probably has a similar metabolic basis (Sternburg & Kearns, 1952).

* Part 6: Kikal & Smith (1959).

The metabolites of DDT in some insects are still not known and, as well as 2:2-bis-(*p*-chlorophenyl)-1:1-dichloroethylene (DDE), cockroaches excrete five unidentified metabolites which account for most of the dose (Robbins & Dahm, 1955; Lindquist & Dahm, 1956). Flies (Terriere & Schonbrod, 1955) and lice (Perry & Buckner, 1958) also excrete much of a dose of DDT as unidentified water-soluble material, which with flies appeared to be mainly phenolic in nature although part was not extractable from acid solution.

Detoxication mechanisms are also important in the resistance of some insects to γ-hexachlorocyclohexane (Gammexane) (Openoorth, 1955), and a complex mixture of water-soluble metabolites is formed by the degradation of this insecticide in flies (Bradbury, 1957). It is possible that some of these water-soluble products are cysteine derivatives like the mercapturic acids formed in the metabolism of chlorinated compounds in vertebrates (Williams, 1947). Mercapturic acid or arylcysteine formation in insects appears to have been recorded only by Kikal & Smith (1958), but the identification of a number of derivatives of dichlorothiophenols from flies dosed with isomers of benzene hexachloride suggests that a cysteine conjugation may be involved in the metabolism of these compounds (Bradbury & Standen, 1959). We have therefore tested for the presence of a detoxication mechanism involving cysteine conjugation by dosing locusts with [^{14}C]chlorobenzene.

EXPERIMENTAL

Materials

[^{14}C]*Chlorobenzene*. This was obtained (0·01 mc/mg.) from The Radiochemical Centre (Amersham, Bucks.) and was diluted either with inactive chlorobenzene or with arachis oil to give convenient volumes for injection. Its identity and purity (> 99·5%) were checked by comparing the activity of such solutions with that of the *p*-chlorobenzenesulphonamide prepared from them after dilution with additional inactive chlorobenzene (see below).

Reference compounds. L-*p*-Chlorophenylmercapturic acid, m.p. 153°, *p*-chlorophenyl-L-cysteine, m.p. 191° (decomp.), L-*m*-chlorophenylmercapturic acid, 165°, *m*-chlorophenyl-L-cysteine, 183° (decomp.), and *o*-chlorophenyl-L-cysteine, m.p. 180° (decomp.), were prepared according to Parke (1955) and Parke & Williams (1951). The cysteine derivatives were more conveniently purified from cystine via their hydrochlorides by crystallizing crude chlorophenyl-L-cysteines from the minimum amount of 2N-HCl and drying over KOH *in vacuo*. *p-Chlorophenyl-L-cysteine hydrochloride* formed colourless plates, m.p. 205° (decomp.); $[\alpha]_D^{30}$ +15 in 0·1N-NaOH (*c*, 1) (Found: HCl, 13·6%). *m-Chlorophenyl-L-cysteine hydrochloride* formed colourless needles, m.p. 180° (decomp.); $[\alpha]_D^{30}$ +13 in 0·1N-NaOH (*c*, 1) (Found: HCl, 13·1%). *o-Chlorophenyl-L-cysteine hydrochloride* formed colourless plates, m.p. 190° (decomp.); $[\alpha]_D^{30}$ +8 in 0·1N-NaOH (*c*, 1) (Found HCl, 13·6. $C_9H_{10}O_2NClS \cdot HCl$ requires HCl, 13·6%). All isomers were moderately soluble in cold water and very soluble in ethanol.

Methods

Locusts and dosing. *Schistocerca gregaria* were obtained from the Anti-Locust Research Centre as immature adults and were stored under the conditions recommended by Hunter-Jones (1956). Compounds were administered by injection into the thorax with an Agla micrometer syringe as solutions (0·1–0·05 ml.) in arachis oil or water. Unless the respired air was to be collected, the dosed locusts were kept singly in paper-covered 500 ml. beakers at 25–30°. Dissection of the insects was carried out on a wax block sprinkled with solid CO_2 to minimize loss of any volatile metabolite and the dissected tissues were weighed and stored in glass-stoppered bottles at −40°.

Measurement of radioactivity. All measurements were carried out with thin end-window-counter tubes, the background of which was 15–18 counts/min., and specific activities were determined by comparison with a 0·1 μC/g. polymer reference sample (The Radiochemical Centre) which counted at about 100 counts/min. over background. Solid samples were counted on 1·5 cm.^{2} planchettes in 'infinitely thick' layers, and liquids and homogenates in inverted planchettes, which were referred to similarly prepared plastic standards. Counts were made for times sufficient to give S.E. ±5% (cf. Calvin, Heidelberger, Reid, Tolbert & Yankwich, 1949). Tissues and excreta were usually mixed with a small known volume of water or ethanol in a glass Potter homogenizer and counted as thick homogenates, but in some cases 10–20 mg. of tissue samples were oxidized to CO_2 by the Van Slyke & Folch (1940) procedure and counted as $BaCO_3$. No significant difference was found in results obtained by the two techniques.

Paper chromatography. This was carried out as described earlier (Smith, Smithies & Williams, 1953) and approximate R_F values are quoted in Table 1. No solvents were found to

Table 1. *R_F values of chlorophenyl-mercapturic acids and -cysteines*

Whatman no. 4 paper and the descending method were used. Solvent mixtures, with times of run in parentheses, were: A, butan-1-ol–acetic acid–water (4:1:5, by vol.) (7 hr.); B, methyl ethyl ketone–water–2N-acetic acid (200:100:1, by vol.) (2 hr.); C, methyl ethyl ketone saturated with water (2 hr.); D, benzene–butan-1-ol–acetic acid–water (1:1:1:5, by vol.) (7 hr.); E, methyl ethyl ketone–aq. 2N-NH_3 soln. (1:1, v/v) (2 hr.).

	R_F				
Solvent mixture	A	B	C	D*	E*
o-, *m*- and *p*-Chlorophenylmercapturic acid	0·9	0·8	0·5	0·8	0·2
o-, *m*- and *p*-Chlorophenyl-L-cysteine	0·7	0·3	0·3	0·5	0·2

* *para*Isomer only.

separate satisfactorily the three isomers of either chlorophenylcysteine or chlorophenylmercapturic acid. The chlorophenylcysteines were detected by the purple colour formed when the paper was sprayed with 0·1% (w/v) butan-1-ol solution of ninhydrin and heated to 100° for 10 min. They also quenched the blue fluorescence of filter paper under illumination with a filtered low-pressure Hg arc (Hanovia Chromatolite). Chlorophenyl-cysteines and -mercapturic acids were both detected by the $Ag_2Cr_2O_7$ reagent of Knight & Young (1958). Normal crop fluid and excreta of locusts contained large amounts of ninhydrin-reacting materials but these had R_F values smaller than those of any of the cysteine conjugates in the solvents used.

Spectrophotometric estimation of p-*chlorophenylmercapturic acid.* Samples of the ethanolic suspension of excreta containing 50–300 μg. of *p*-chlorophenylmercapturic acid were separated by paper chromatography in solvent system B (Table 1). The zone containing the mercapturic acid, located on a reference strip by the $Ag_2Cr_2O_7$ reagent, was cut out and extracted by keeping with occasional shaking in 10 ml. of ethanol for 1 hr. The *p*-chlorophenylmercapturic acid in the extract was estimated by measurement of the absorption at 260 mμ in a Unicam SP. 500 spectrophotometer and comparison with a calibration curve prepared from known amounts of *p*-chlorophenylmercapturic acid similarly separated and extracted. Extracts of paper blanks showed no absorption at this wavelength and recoveries of *p*-chlorophenylmercapturic acid (50–300 μg.) added to normal locust excreta were within ±10 μg.

Estimation of p-*chlorophenyl-*L-*cysteine.* (*a*) Ninhydrin method. The *p*-chlorophenyl-L-cysteine (40–100 μg.) was separated from other ninhydrin-reacting material in excreta by chromatography in solvent A or B as described for the mercapturic acid above. Zones containing *p*-chlorophenylcysteine, located by the fluorescence-quenching effect, were cut out and treated with 1 ml. of 0·1 N-NaOH. After drying over H_2SO_4 *in vacuo* overnight to remove NH_3, the excess of alkali was neutralized (Fowden, 1951) and the amino acid on the paper estimated by the procedure of Yemm & Cocking (1953), the paper being immersed in the reagent solution. Amounts of *p*-chlorophenyl-L-cysteine (40–160 μg.) added to normal locust excreta were recovered within 5 μg. when estimated by this procedure. *m*-Chlorophenylcysteine gave a calibration curve identical with that of the *p*-isomer. (*b*) Fluorimetric method. *p*-Chlorophenyl-L-cysteine fluoresced in ultraviolet light (see Table 2). The relation between fluorescence and concentration in 0·1 N-NaOH was linear from 1 to 6 μg./ml., the lower limit being governed by the background light scatter of the solutions. Solutions of higher concentration, where concentration quenching occurred, were measured after dilution to bring the concentration within the linear range. No fluorescence of the *o*- or *m*-isomers was detected in the concentration range 1–100 μg./ml. at the instrument settings used to measure the *p*-isomers. The *p*-chlorophenylcysteine (5–30 μg.) was separated from mercapturic acids and other fluorescing substances by paper chromatography in solvent A or B and the papers were dried in a current of air for at least 4 hr. The appropriate zones were located and cut out and the chlorophenylcysteine was extracted from the paper by shaking for 30 min. in 5 ml. of 0·1 N-NaOH. The solution was filtered through washed glass wool to remove paper fibres, and the fluorescence of the solution was compared with a standard solution (5 μg./ml.) of *p*-chlorophenylcysteine in 0·1 N-NaOH at 360 mμ in an Aminco–Bowman spectrophotofluorimeter with exciting wavelength set to 280 mμ. Recoveries of *p*-chlorophenyl-L-cysteine (10–100 μg.) added to normal locust excreta were within ±2 μg.

Distribution of radioactivity in locust

Excretion of unchanged chlorobenzene. Locusts were injected with [^{14}C]chlorobenzene (0·05–0·5 mg.) and placed in a 500 ml. flask through which a slow stream of air, washed through 2 N-NaOH, was drawn. The air leaving the flask was drawn through a drying tube containing magnesium perchlorate, through two tubes, each containing 10 ml. of ethanol, cooled to −50°, and finally through 2 N-NaOH.

Table 2. *Instrumental values of fluorescence of some metabolites of chlorobenzene*

Readings are the product of meter multiplier and deflexion in the Aminco–Bowman spectrophotofluorimeter. Solutions contained 5 μg./ml.

	In 0·1 N-NaOH			In 0·1 N-HCl		
	Exciting wavelength (mμ)	Fluorescence wavelength (mμ)	Reading	Exciting wavelength (mμ)	Fluorescence wavelength (mμ)	Reading
p-Chlorophenylcysteine	280	360	1·35	280	340	0·81
p-Chlorophenylmercapturic acid	270	350	0·51	270	360	0·90

Table 3. *Radioactivity recovered after injection of* [^{14}C]*chlorobenzene into locusts*

No. of locusts	Chlorobenzene injected (mg./locust)	Percentage of radioactivity found after 48 hr. in		
		Expired air	Excreta	Locust
1*	0·05	51	No excreta	49
1*	0·05	49	1	50
2	0·05	40	29	31
1	0·35	53	33	14
4	0·45	35	44	21
6	0·6	18	30	52

* 24 hr. experiment.

The ethanol tubes were removed periodically and the ^{14}C was assayed by liquid counting. At no time was more than a trace of activity found in the second ethanol tube. Control experiments showed that 0·2–1·0 mg. of [^{14}C]-chlorobenzene was quantitatively recovered in this apparatus in 6 hr.

After 48 hr. the excreta were removed and the flask was rinsed out with a small volume of ethanol; the excreta and washings were mixed in an Ato-Mix homogenizer to form a thick suspension which was assayed for ^{14}C (see Table 3). The radioactivity collected in the cold ethanol trap was pooled and, by dilution analysis (see below), was shown to be all in the form of chlorobenzene. The CO_2 trapped in the final NaOH wash-bottle was precipitated as $BaCO_3$ and assayed for ^{14}C. In two experiments with high-activity [^{14}C]chlorobenzene less than 0·1% of the dose was found as CO_2.

Radioactivity in locust organs. Immature adult locusts were each dosed with 2 mg. (0·2 μc) of [^{14}C]chlorobenzene and kept at 25–30°. At intervals one locust was removed and dissected and the radioactivity measured in the fat, muscle, foregut, hindgut, heart, integument and excreta. A small accumulation of radioactivity appeared to take place in the fat which was at a maximum 8 hr. after dosing, when 15% of the total dose was found in this site. No significant activity appeared to accumulate in any other sample tested except the hindgut and excreta, where the radioactivity increased progressively. In other experiments some variation was found in the rate at which the radioactivity appeared in the excreta (Table 3).

Radioactivity in excreta. A sample of freshly voided excreta, containing 0·66 μc, from locusts dosed with [^{14}C]chlorobenzene was heated for 12 hr. at 100° in a sealed tube with 10 ml. of N-HCl. After cooling, the solution was partitioned into neutral (15·9%), alkali-soluble (61·7%) and acid-soluble (20·4%) fractions as shown in Table 4. The neutral fraction (15·9%) was shown by dilution with inactive chlorobenzene and counting as *p*-chlorobenzenesulphonamide (see below) to consist only of chlorobenzene. The alkali-soluble fraction appeared to consist mainly of phenols (Gessner & Smith, 1960). Further continuous extraction of the acid-soluble fraction with ether removed no more radioactivity.

Estimations by isotopic dilution

The radioactivity in excreta was partly present in an insoluble form which could be removed from the fibrous food residues only by repeated washing with ethanol or N-NaOH. The thick homogenate of the excreta, after the radioactivity had been counted, was therefore diluted with ethanol to give about a 1–5% (w/v) suspension and samples for dilution analysis were withdrawn from this after shaking well. Inactive compounds were usually dissolved in a volume of this suspension such that, after isolation again, a metabolite accounting for 1% of the activity counted at not less than 5 counts/min. After purification to constant specific activity the isolated material was normally converted into a second derivative, and this, after purification, was shown to have the same equivalent specific activity as the first. Results quoted in Table 5 are calculated from two such concordant values.

Chlorobenzene. The ethanolic solution containing [^{14}C]-chlorobenzene was mixed with five times its volume of water and 1 g. of inactive chlorobenzene. The mixture was then extracted twice with an equal volume of ether. After drying over $CaCl_2$ the ether was evaporated and chlorosulphonated as described by Huntress & Carten (1940). The *p*-chlorobenzenesulphonyl chloride, m.p. 48°, was counted after crystallization from dry ether and converted into *p*-chlorobenzenesulphonamide, m.p. 144° (Huntress & Carten, 1940). This was crystallized to constant activity from aqueous ethanol.

p-*Chlorophenyl*-L-*cysteine.* The non-radioactive compound (0·2–0·5 g.) was added to a suitable volume of radioactive ethanolic suspension or homogenate and heated with 20–50 ml. of 0·5 N-H_2SO_4 until solution was complete. After filtration from grass or tissue debris, the filtrate was washed with 2 × 25 ml. of ether and 2 × 25 ml. of $CHCl_3$ and adjusted to pH 4·0–5·0. The precipitated *p*-chlorophenylcysteine was recrystallized from ethanol–water (1:1, v/v) to constant m.p. (191°, decomp.) and constant specific activity. The purified product was recrystallized from N-HCl to give *p*-chlorophenyl-L-cysteine hydrochloride, m.p. 205° (decomp.), with the same equivalent specific activity.

L-p-*Chlorophenylmercapturic acid.* The radioactive solution or ethanolic homogenate was mixed with non-radioactive L-*p*-chlorophenylmercapturic acid (0·2 g.), 25 ml. of 2 N-HCl and 25 ml. of ethanol and warmed until solution was complete. After removal of any grass residues from the excreta by filtration, the solution was extracted with 5 × 100 ml. of $CHCl_3$ and the mercapturic acid re-extracted from the pooled $CHCl_3$ layers with 10 ml. of 2 N-NaOH. The alkaline solution was acidified to pH 3·0 and the precipi-

Table 4. *Partition of radioactivity of hydrolysed excreta from locusts dosed with* [^{14}C]*chlorobenzene*

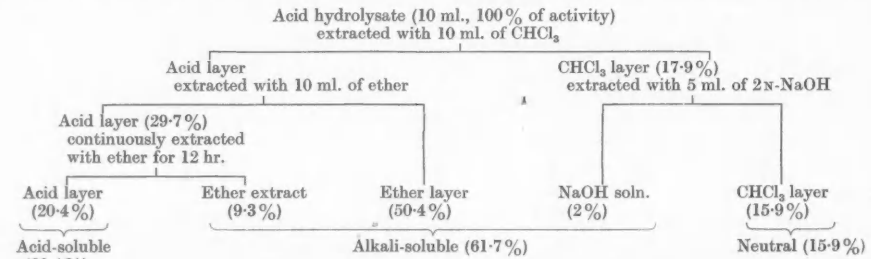


Table 5. *Excretion in 48 hr. of some metabolites of* [^{14}C]*chlorobenzene by locusts*

—, Not examined.

No. of locusts used	1	5	1	4	6
Dose of chlorobenzene (mg./locust)	0·05*	0·3	0·35	0·45	0·6
Metabolite excreted	Percentage of dose				
Chlorobenzene					
In expired air	49·5	—	48·7	30·1	18·0
In excreta (first day)	—	3·9	1·7	—	—
In excreta (second day)	—	—	0·6	—	—
Left in locust	—	—	9·8	—	29·0
p-Chlorophenylcysteine					
In excreta (first day)	—	—	3·7	2·3	1·4
In excreta (second day)	—	—	0·3		
Left in locust	4·5	—	<1·0	.	.
p-Chlorophenylmercapturic acid					
In excreta (first day)	—	—	0·7	0·8	0·3
In excreta (second day)	—	—	<0·2		
Left in locust	—	—	<0·1	.	.
m-Chlorophenylcysteine in excreta	—	0·3	—	3·0	0·6
m-Chlorophenylmercapturic acid in excreta	—	—	—	—	0·1
o-Chlorophenylcysteine in excreta	—	—	—	—	0·4

* This locust provided no excreta.

Table 6. *Cysteine conjugates in excreta of locusts dosed with* [^{14}C]*chlorobenzene*

Pooled excreta from six locusts given 0·6 mg. of [^{14}C]-chlorobenzene each were used for each experiment.

Metabolite	Percentage of total radioactivity of excreta: Without acidification	After heating with N-HCl
m-Chlorophenyl-L-cysteine	<0·5	2·2
L-*m*-Chlorophenylmercapturic acid	<0·2	0·3
p-Chlorophenyl-L-cysteine	1·0	5·0
L-*p*-Chlorophenylmercapturic acid	<0·2	1·2

tated mercapturic acid recrystallized from ethanol–water (1:1, v/v) to constant m.p. (152°) and specific activity. The purified mercapturic acid (0·1 g.) was boiled with 20 ml. of N-NaOH for 2 hr. A portion (10 ml.) of 2N-H_2SO_4 was then added and the solution titrated with 0·05N-I_2 solution until precipitation of *pp'*-dichlorodiphenyl sulphide was complete (cf. Parke, 1955). The disulphide (m.p. 71°) after washing with water and drying had the same equivalent specific activity as the mercapturic acid.

m-*Chlorophenyl-L-cysteine.* A suitable volume of the ethanolic suspension of excreta was diluted with water and 0·5 g. of non-radioactive *m*-chlorophenyl-L-cysteine added. The solution was made N to HCl, heated to 80–90° and cooled immediately. The precipitate formed on neutralization with 2N-NaOH was reprecipitated three times by solution in N-NaOH and acidification with 2N-acetic acid and then crystallized from 5N-HCl to constant activity. The *m*-chlorophenyl-L-cysteine hydrochloride was converted into the free base, m.p. 183° (decomp.), and had the same equivalent specific activity as the hydrochloride.

L-m-*Chlorophenylmercapturic acid.* The non-radioactive material (0·5 g.) was added to the ethanolic suspension of excreta with water and the solution made N to HCl. The solution was heated until all the added material was dissolved, and cooled. The precipitate was purified by solution in N-NaOH and reprecipitation with 2N-acetic acid and then crystallized to constant specific activity and m.p. (165°) from 50% (v/v) ethanol.

o-*Chlorophenyl-L-cysteine.* This was isolated again as described for the *m*-isomer and counted as the hydrochloride, m.p. 190° (decomp.), and as *o*-chlorophenylcysteine, m.p. 180° (decomp.).

Precursors of the chlorophenyl-cysteines and -mercapturic acids

Excreta were collected for 2 days after dosing locusts with [^{14}C]chlorobenzene and were homogenized in ethanol. Suitable volumes of this suspension were added to non-radioactive samples (0·5 g.) of *m*- and *p*-chlorophenyl-L-cysteine and *m*- and *p*-chlorophenylmercapturic acid which had been dissolved in 1 mol.prop. of NaOH and diluted to 20 ml. with water. The solutions were immediately acidified with 2N-acetic acid and the precipitates collected. These were purified by twice precipitating from solution in 0·1N-NaOH with 2N-acetic acid and then crystallized as described above until the specific activity was constant or not significantly different from the background.

A second set of estimations on this sample of excreta was carried out as described above except that the solutions were heated for a few seconds at 80° after making N to HCl, and quickly cooled before isolation of the compounds. The results of these analyses, quoted in Table 6, show that only small amounts of mercapturic acid or cysteines are present in excreta before acid treatment. It was concluded from these results that both the mercapturic acids and chlorophenylcysteines estimated in locust excreta are excreted as precursors similar to the premercapturic acids found in vertebrates (Knight & Young, 1958; Boyland & Sims, 1958).

Absence of a chlorobenzene precursor. An aqueous suspension of freshly voided excreta, from locusts dosed with [^{14}C]chlorobenzene, which contained 0·58 μc, was divided into two equal parts and non-radioactive chlorobenzene (0·5 g.) was added to each. One part was immediately extracted with ether and the chlorobenzene recovered and converted into *p*-chlorobenzenesulphonamide as described above. The second portion was heated in a sealed tube at 100° for 12 hr. with N-HCl and the chlorobenzene was extracted and converted into *p*-chlorobenzenesulphonamide. After crystallization to constant activity the first preparation carried 0·068 μc and the second 0·069 μc, and it was concluded that an acid-labile precursor (cf. Bourne & Young, 1934) of chlorobenzene was not a significant metabolite of chlorobenzene in the locust.

Fate of the cysteine conjugates in locusts

In most species, mercapturic acids appear to be the main cysteine conjugate excreted and it was thought possible that the preponderance of arylcysteines in locust excreta might be due to the action of an acylase in the gut which hydrolysed the initial detoxication product (cf. Kikal & Smith, 1959). We have therefore attempted to measure the apparent acylase activity in gut and crop fluid with *p*-chlorophenylmercapturic acid as substrate.

Enzymic hydrolysis of L-p-*chlorophenylmercapturic acid.* The sources of enzyme were crop fluid (cf. Robinson, Smith & Williams, 1953) diluted tenfold with water, or excreta ground in a mortar with four times the weight of water and squeezed through cheesecloth. In the estimation of enzyme activity 0·5 ml. of enzyme, 0·25 ml. of substrate (*p*-chlorophenylmercapturic acid and 1 mol.prop. of NaOH in aqueous solution) and 0·25 ml. of buffer, pH 3·0–8·0 (prepared from 0·2 M-Na_2HPO_4–0·1 M-citric acid) were incubated at 37° for 18–36 hr. Control tubes were prepared (*a*) in which the substrate was added at the end of the incubation period, and (*b*) in which the enzyme was added at the end of the incubation period. Controls (*b*) showed that no hydrolysis of mercapturic acid occurred at any pH in the absence of enzyme, but in controls (*a*) some changes, visible on the paper chromatograms, occurred in the large amounts of ninhydrin-reacting compounds present in the enzyme extracts. After incubation and completion of the control tubes, 0·1 ml. of mixture was withdrawn and separated on Whatman no. 4 paper in solvent system B and the amount of chlorophenylcysteine present was determined by one of the procedures described above. Optimum enzymic hydrolysis of mercapturic acid to *p*-chlorophenylcysteine was found at pH 6·0 in both crop fluid and excreta. The degree of hydrolysis increased with increasing substrate concentration up to a limit set by the solubility of the substrate at pH 6·0 (0·025 M).

When freshly voided excreta or crop fluid were assayed under the optimum conditions of pH and substrate concentration the activity found was of the order of 100 μg./hr./g. of excreta or crop fluid. Control experiments showed, however, that *p*-chlorophenyl-L-cysteine was itself destroyed under these conditions. When 1–2 mg. of *p*-chlorophenylcysteine was incubated at pH 6·0 with 0·5 ml. of the enzyme extracts used for acylase activity, 70–100 % was destroyed in 48 hr.

Fate of injected p-*chlorophenylmercapturic acid and* p-*chlorophenyl*-L-*cysteine.* *p*-Chlorophenylmercapturic acid (2 mg.) was injected into locusts as its sodium salt in 0·1 ml. of water and excreta were collected for 48 hr. The excreta were homogenized with 2 ml. of 50 % (v/v) aqueous ethanolic 0·05 N-HCl and 0·2 ml. of the liquid was chromatographed in solvent system B; the *p*-chlorophenyl-cysteine and -mercapturic acid were estimated as described above. No *p*-chlorophenylcysteine could be detected and 66–79 % of the dose of the mercapturic acid was recovered. No further mercapturic acid was found in the excreta collected over the next 3 days.

When *p*-chlorophenyl-L-cysteine (0·5–1·0 mg.) was similarly injected and the 48 hr. excreta were extracted as described above less than 2 % of the dose appeared as the mercapturic acid, and recoveries of the *p*-chlorophenylcysteine ranged from 9·5 to 12·5 % of the dose. No further material was found when excreta were collected for the next 4 days and no *p*-chlorophenylcysteine could be detected chromatographically in the haemolymph.

Nature of the degradation product. *p*-Chlorophenylcysteine (1 mg.) was incubated for 48 hr. with 0·5 ml. of the excreta enzyme at pH 6·0 as described above and the reaction mixture was examined chromatographically. No *p*-chlorophenyl-cysteine or -mercapturic acid was found, but a spot was present with R_F 0·9 in system D and R_F 0·4 in system E, which reacted strongly with $Ag_2Cr_2O_7$ but gave no ninhydrin reaction. Similar spots from unsprayed chromatograms were eluted

with ethanol and examined by paper electrophoresis as described by Smith (1958). The unknown material migrated to the anode at pH values from 3·0 to 12·0, but, unlike *p*-chlorophenylcysteine, it did not migrate at pH 1·0.

DISCUSSION

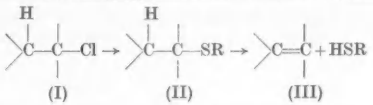
About half of the chlorobenzene injected into the locusts was excreted unchanged, presumably by spiracular respiration, since in one experiment (Table 3) where no excreta were produced 51 % of the dose was recovered by aeration. In other experiments some of the chlorobenzene recovered by aeration may have been voided with the excreta, since, when these were examined after being kept in the respiration chamber for 48 hr., up to 14 % of the radioactivity in the excreta was present as unchanged chlorobenzene.

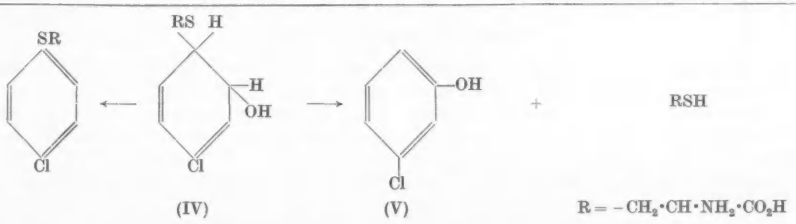
Non-polar fat-soluble substances tend to be retained by the vertebrate kidney and it has been suggested (Brodie & Hogben, 1957) that the detoxication mechanisms of terrestrial vertebrates have developed to change such lipid-soluble material into highly polar, easily excreted products. The orthopteran excretory system, which involves simple diffusion through the Malpighian tubules and water reabsorption in the rectum (Ramsay, 1958), appears, from the excretion of chlorobenzene, to retain lipid-soluble material less effectively. The necessity for locusts to form polar detoxication products may therefore be less pressing than for vertebrates. Formation of the detoxication products was slow and after 48 hr. much of the administered radioactivity still remained in the locust, much of it still as unchanged material.

The cysteine conjugates found in locust excreta were present as acid-labile precursors of chlorophenyl-L-cysteines and -mercapturic acids. This situation is essentially similar to that found in vertebrates. In the vertebrates, however, only the *para*isomer of chlorophenylmercapturic acid has been found as a metabolite of chlorobenzene and a search for other isomers is called for since the presence of both *meta* and *para* isomers in the locust is of interest in the discussion of possible mechanisms for the formation of the premercapturic acids. The absence of any pronounced directive effects of the chlorine in the formation of the cysteine conjugates of chlorobenzene recalls the similar lack of directive effects in some biochemical hydroxylations (Smith, 1950; Gessner & Smith, 1960).

A further difference between the metabolism of chlorobenzene in the locust and in vertebrates is that the chlorophenylcysteines formed are mainly found in the non-acetylated form in the locust, whereas in vertebrates the cysteine conjugates are isolated as their acetyl derivatives, the mercapturic acids. It is not thought likely that the *p*-chlorophenylcysteine found in locust excreta was derived from an acetylated precursor by hydrolysis in the gut (cf. Kikal & Smith, 1959), since the acylase activity in crop fluid and excreta was very low. Moreover, when *p*-chlorophenylmercapturic acid was injected into the locust most of it could be recovered unchanged in the excreta. It is probable that the amounts of chlorophenylcysteines estimated in excreta should be somewhat larger than those actually found since some decomposition occurred in the gut.

As well as the aromatic mercapturic acids and premercapturic acids found in locusts and vertebrates, the latter can also form mercapturic acids by replacement of active halogen atoms such as the chlorine in benzyl chloride (cf. Knight & Young, 1958). It is possible that the sulphur-containing metabolite of Gammexane observed by Bradbury & Standen (1959) is of this type and it is interesting to consider whether a similar cysteine derivative is involved in metabolism of DDT. Glutathione, which could be the source of the cysteine of the mercapturic acids (Bray, Franklin & James, 1959), is required for the metabolism of both Gammexane and DDT *in vitro*, and the former is largely converted into sulphur conjugates *in vivo*. If DDT (I) formed a similar cysteine conjugate (II), a decysteinylation reaction would give the known metabolites (III), DDE from DDT and pentachloro*cyclo*hexene (Bradbury & Standen, 1958) from Gammexane.





In acid solution, premercapturic acids which probably have structures like (IV) (cf. Boyland & Sims, 1958), readily undergo a reaction similar to this postulated decysteinylation and, if this reaction were accomplished by an enzyme (cf. Knight & Young, 1958), it could be a source of the phenols (e.g. V) found as metabolites of chlorobenzene in locusts (Gessner & Smith, 1960). In this case the *m*-chlorophenol would arise from the same precursor as the *p*-chlorophenylcysteine.

SUMMARY

1. The excretion and metabolism of [^{14}C]chlorobenzene has been studied in the locust *Schistocerca gregaria*.

2. About half of the dose was excreted unchanged.

3. The probable presence, in excreta, of *o*-, *m*- and *p*-chlorophenyl-L-cysteine and smaller amounts of *m*- and *p*-chlorophenylmercapturic acid was shown by isotopic-dilution techniques.

4. These compounds were excreted as acid-labile precursors.

5. A weak acylase is present in crop fluid and excreta which slowly hydrolyses L-*p*-chlorophenylmercapturic acid to *p*-chlorophenyl-L-cysteine.

6. *p*-Chlorophenyl-L-cysteine is enzymically degraded to unidentified products by crop fluid and excreta.

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REFERENCES

Bourne, M. C. & Young, L. (1934). *Biochem. J.* **28**, 803.
Boyland, E. & Sims, P. (1958). *Biochem. J.* **68**, 440.
Bradbury, F. R. (1957). *J. Sci. Fd Agric.* **8**, 90.
Bradbury, F. R. & Standen, H. (1958). *J. Sci. Fd Agric.* **4**, 203.
Bradbury, F. R. & Standen, H. (1959). *Nature, Lond.*, **183**, 983.
Bray, H. G., Franklin, T. J. & James, S. P. (1959). *Biochem. J.* **71**, 690.
Brodie, B. B. & Hogben, C. A. M. (1957). *J. Pharm., Lond.*, **9**, 345.
Calvin, M., Heidelberger, C., Reid, J. C., Tolbert, B. M. & Yankwich, P. (1949). *Isotopic Carbon*. London: Chapman and Hall.
Fowden, L. (1951). *Biochem. J.* **48**, 327.
Gessner, T. & Smith, J. N. (1960). *Biochem. J.* **75**, 172.
Hunter-Jones, P. (1956). *Instructions for Rearing and Breeding Locusts*. London: Anti-Locust Research Centre.
Huntress, E. H. & Carten, F. H. (1940). *J. Amer. chem. Soc.* **62**, 512.
Kikal, T. & Smith, J. N. (1958). *Biochem. J.* **69**, 52P.
Kikal, T. & Smith, J. N. (1959). *Biochem. J.* **71**, 48.
Knight, R. H. & Young, L. (1958). *Biochem. J.* **70**, 111.
Lindquist, D. A. & Dahm, P. A. (1956). *J. econ. Ent.* **49**, 579.
Metcalf, R. L. (1955). *Physiol. Rev.* **35**, 197.
Openoorth, F. J. (1955). *Nature, Lond.*, **175**, 124.
Parke, D. V. (1955). *Biochem. J.* **59**, 422.
Parke, D. V. & Williams, R. T. (1951). *Biochem. J.* **48**, 624.
Perry, A. S. & Buckner, A. J. (1958). *Amer. J. trop. Med. Hyg.* **7**, 620.
Ramsay, J. A. (1958). *J. exp. Biol.* **35**, 871.
Robbins, W. E. & Dahm, P. A. (1955). *Agric. Fd Chem.* **3**, 500.
Robinson, D., Smith, J. N. & Williams, R. T. (1953). *Biochem. J.* **53**, 125.
Smith, J. N. (1950). *Symp. biochem. Soc.* **5**, 15.
Smith, J. N. (1958). *Biochem. J.* **69**, 509.
Smith, J. N., Smithies, R. H. & Williams, R. T. (1953). *Biochem. J.* **54**, 225.
Sternburg, J. & Kearns, C. W. (1952). *J. econ. Ent.* **45**, 497.
Terriere, L. C. & Schonbrod, R. D. (1955). *J. econ. Ent.* **48**, 736.
Van Slyke, D. D. & Folch, J. (1940). *J. biol. Chem.* **136**, 509.
Williams, R. T. (1947). *Detoxication Mechanisms*. London: Chapman and Hall.
Yemm, E. W. & Cocking, E. C. (1953). *Analyst*, **80**, 209.

Biochem. J. (1960) **75**, 172

Comparative Detoxication

8. THE METABOLISM OF CHLOROBENZENE IN LOCUSTS: PHENOLIC METABOLITES, A COMPARISON WITH SOME VERTEBRATE SPECIES

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Oxidation of foreign aromatic compounds is one of the most common detoxication mechanisms found in vertebrates, but whether this process occurs to any significant extent in insects is not yet clear. Some examples of the hydroxylation of aromatic rings in insects are known but these mainly concern the oxidation of naturally occurring substrates involved in amino acid metabolism or cuticle tanning (Richards, 1951; Smith, 1955; Dennell, 1958). There is also some evidence that some insecticides may be oxidized to phenols in insects (Terriere & Schonbrod, 1955; Bradbury & Standen, 1958), but the identity of the metabolites is not known.

In vertebrates the hydroxylation of natural aromatic substrates appears to be carried out by specific enzyme systems which differ from that which carries out the detoxication oxidations (Mitoma, Posner, Reitz & Udenfriend, 1956). Brodie & Hogben (1957) have suggested that the development of this detoxication system by terrestrial vertebrates is connected with their need to produce polar, water-soluble metabolites capable of excretion by the kidney.

It was of interest to see whether an insect had developed a similar oxidation system producing a similar pattern of phenolic metabolites to those known in vertebrates. Chlorobenzene was chosen for testing for the oxidation system in the locust as its metabolism has been well studied in vertebrates (Smith, Spencer & Williams, 1950; Azouz, Parke & Williams, 1952, 1953) and because of its relation to a number of widely used insecticides.

EXPERIMENTAL

Materials

[^{14}C]*Chlorobenzene*. This was obtained (0·01 mc/mg.) from The Radiochemical Centre (Amersham, Bucks) and was normally dissolved in a suitable volume of arachis oil before use, as described by Gessner & Smith (1960).

Reference compounds. *m*- and *p*-Chlorophenol, 2-chloroquinol and 4-chlororesorcinol were commercial samples. Commercial samples of *o*-chlorophenol contained phenol and were purified according to Wohlleben (1909). 4-Chlorocatechol was prepared according to Peratoner (1898) and purified from 3-chlorocatechol via its toluene-*p*-sulphonate (Smith *et al.* 1950). The 4-chlorocatechol obtained by alkaline hydrolysis of this derivative had m.p. 92° and was chromatographically homogeneous. 2-Chlororesorcinol (m.p. 97°) was prepared according to Milligan & Hope (1941).

The potassium salts of *o*-, *m*- and *p*-chlorophenyl hydrogen sulphate were prepared according to Burkhardt & Lapworth (1926). *o*-Chlorophenyl β-glucoside (m.p. 170°; $[\alpha]_D^{25} - 90°$), *m*-chlorophenyl β-glucoside (m.p. 179°; $[\alpha]_D^{25} - 36°$) and *p*-chlorophenyl β-glucoside (m.p. 175°; $[\alpha]_D^{25} - 87°$) were prepared by the method of Glazer & Wulwek, 1924. L-Chlorophenylmercapturic acids and chlorophenyl-L-cysteines were samples used previously (Gessner & Smith, 1960).

Methods

Animals and dosing. Locusts (*Schistocerca gregaria*) were obtained and kept, and excreta and locust bodies were homogenized and stored as an ethanolic suspension as previously described (Gessner & Smith, 1960).

Rabbits, cats, rats and ferrets were kept in cages over metal funnels to collect urine and chlorobenzene was given orally by stomach tube (rabbits and rats) or in gelatin capsules (cats and ferrets). Urines from each species were collected until they appeared to be free from chlorophenols and were pooled. With cats it was necessary to collect the urine for at least 4 days.

Measurement of radioactivity. This was carried out as described previously (Gessner & Smith, 1960).

Paper chromatography. This was carried out as described by Smith, Smithies & Williams (1953) and approximate R_F values are quoted in Table 1. Satisfactory separations of the three isomeric chlorophenols or their conjugates were obtained only in systems A and B. In separations in solvent system A, the Whatman no. 4 filter paper was soaked in 0·2 N-Na_2CO_3 and squeezed between sheets of blotting paper with a hard-rubber roller to press out as much liquid as possible. Solutions of chlorophenols were then applied in ether or methanol, and the paper was dried in air for a few minutes before developing in *n*-hexane containing 10% (v/v) of *iso*propyl ether. If the aqueous phase was sprayed on to the paper before use it was more difficult to obtain level fronts.

For separations in system B, the paper was soaked in a 20% (v/v) solution of formamide in methanol and pressed between blotting paper as described above. Solutions were

Table 1. *R_F values of some possible metabolites of chlorobenzene*

Solvent mixtures, with time of run in parentheses, were: A, hexane–*iso*propyl ether (10:1, v/v) on paper treated with 0·2 N-Na_2CO_3 (1·5 hr.); B, hexane on paper treated with formamide (1·5 hr.); C, butan-1-ol–acetic acid–water (4:1:5, by vol.) (7 hr.); D, butan-1-ol saturated with water (7 hr.); E, benzene–acetic acid–water (1:1:2, by vol.) (3 hr.); F, pyridine–benzene–acetic acid–water (3:1:5:3, by vol.) (7 hr.); G, butan-1-ol–benzene–acetic acid–water (1:1:1:5, by vol.) (7 hr.); H, hexane–*iso*propyl ether (5:1, v/v) on paper treated with glycerol (1·5 hr.). Whatman no. 4 paper and the descending method were used.

	R_F							
Solvent mixture	A	B	C	D	E	F	G	H
o-Chlorophenol	0·3	0·5	1·0	0·9	0·8	1·0	1·0	0·9
m-Chlorophenol	0·5	0·7	1·0	0·9	0·8	1·0	1·0	0·9
p-Chlorophenol	0·6	0·6	1·0	0·9	0·8	1·0	1·0	0·9
o-, *m*- or *p*-Chlorophenylglucoside	0	—	0·8	0·8	0	0·8	0·6	—
o-, *m*- or *p*-Chlorophenylsulphate	0	—	0·6	0·4	0	0·6	0·2	—
o-, *m*- or *p*-Chlorophenylcysteine	0	—	0·7	0·6	0	0·6	0·5	—
o-, *m*- or *p*-Chlorophenylmercapturic acid	—	—	0·9	0·5	0·7	0·6	1·0	—
4-Chlorocatechol	0	0·1	1·0	—	0·3	0·9	1·0	0·2
4-Chlororesorcinol	0	—	0·9	—	0·2	0·9	0·9	—
2-Chlororesorcinol	0	—	0·9	—	0·25	0·9	—	0·03
2-Chloroquinol	0	—	0·9	—	0·2	0·9	0·9	0·03
Phenol	0·4	0·3	—	—	0·9	—	—	0·6

applied in ether or methanol and the papers dried in air until the smell of methanol was no longer detectable (about 10–15 min.). Papers were then developed in *n*-hexane. Separations were satisfactory with this system only when Whatman no. 4 paper was used. No separation resulted when methanol was omitted and papers were treated with pure formamide. System H was prepared in the same way as B except that glycerol replaced formamide and *n*-hexane–*iso*propyl ether (5:1, v/v) was used as the mobile phase.

Detection of compounds on chromatograms. Phenols were detected by spraying with ethanolic 0·01 % dichloroquinonechloroimide followed by a spray of saturated aq. $NaHCO_3$. Chloromonophenols and 4-chlorocatechol gave blue colours and 2- and 4-chlororesorcinol gave purple colours. 2-Chloroquinol gave a green–brown spot which soon faded to brown. 4-Chlorocatechol and 2-chloroquinol gave immediate black spots when sprayed with 0·1 N-$AgNO_3$ containing 1 % (v/v) of aq. NH_3 soln. (sp.gr. 0·88). Other polyphenols reacted in a few minutes with this reagent to give brown spots. Chloroquinol and 4-chlororesorcinol, which had the same R_F, could be distinguished by these two reactions.

Glucosides were located by spraying the papers with a 1:5 (v/v) dilution of *Helix pomatia* gastric juice, which is rich in β-glucosidase, followed 5 min. later by the dichloroquinonechloroimide reagent to detect liberated phenols. Ethereal sulphates were detected by hydrolysis with a spray of 0·1 N-HCl followed by heating to 50–60° for 10 min. and detection of the liberated phenols. Chlorophenylcysteines and chlorophenylmercapturic acids were detected as described by Gessner & Smith (1960).

Chromatography of [^{14}C]*compounds.* Paper chromatograms were run in the usual way on Whatman no. 4 paper and reference compounds were normally mixed with about 0·1 μc of the radioactive extracts so that radioautographs could be compared with the colorimetric location of reference spots. Radioautographs were obtained by leaving the developed chromatograms in contact with X-ray film in light-proof cassettes for 14 days. When volatile radioactive phenolic material was present on the chromatograms the whole film was lightly fogged on development, and though attempts were made to minimize this effect by spraying the chromatograms with 2 N-Na_2CO_3 before radioautography, it was apparent that some diffusion and loss of phenols still occurred.

The radioactive chromatograms were also scanned under an end-window counter. A thin aluminium mask was made to cover all but a 1 cm. slot across the window of the counter tube and the paper was passed across this, each centimetre being counted for 1 min. Results were plotted graphically and compared with the spots produced by colorimetric detection of reference compounds incorporated into the mixture and with radioautographs. With the procedures used it was observed that a peak scanning at 20 counts/min. over background did not darken the X-ray film after 14 days.

Separation of chlorophenols on partition columns. Hyflo Supercel (Johns–Manville Co.) was stirred with an equal weight of aq. 0·2 N-Na_2CO_3 until an even dry mix was obtained. This was made into a slurry with *n*-hexane and packed into a column 2·5 cm. × 100 cm. by lightly consolidating the filling with a wooden pestle. Columns usually allowed 2–3 drops of hexane/sec. to flow through and no better separation was found when tighter packing was used. Mixtures of *o*-, *m*- and *p*-chlorophenol (1–20 mg.) were put on the column in *n*-hexane (10–100 ml.) and development was carried out with *n*-hexane–*iso*propyl ether (15:1, v/v). Fractions of eluate (10 ml.) were collected and the u.v. absorption of each was measured at 280 mμ against a hexane blank to detect the presence of *m*- or *p*-chlorophenol. When *o*-chlorophenol was being eluted, 50 ml. fractions were taken, and since these contained much less phenol it was more convenient to extract the hexane eluates with 4 ml. of 0·1 N-NaOH and to measure the absorption of this at 294 mμ against a 0·1 N-NaOH blank. With columns of the dimensions quoted, *m*- and *p*-chlorophenol appeared as two overlapping bands in the elution chromatogram from 400 to 800 ml. and *o*-chlorophenol from 1200 to 2500 ml. of eluate. Fractions containing *m*- and *p*-chlorophenol or *o*-chlorophenol were pooled and extracted with 6 × 15 ml. of 0·1 N-NaOH; the solutions were diluted to 100 ml. and the extracted phenols estimated colorimetrically as described below.

Estimation of chlorophenols

o-Chlorophenol. The u.v. absorption of solutions in 0·1 N-NaOH was measured at 294 mμ in a Unicam SP. 500 spectrophotometer and the amount of *o*-chlorophenol present was calculated by reference to a calibration curve of similar solutions of *o*-chlorophenol in 0·1 N-NaOH. Recoveries of *o*-chlorophenol (0·5–5·0 mg.) mixed with 10–20 mg. amounts of *m*- and *p*-chlorophenol and separated on partition columns were within the range 80–95 %.

m- *and* p-*Chlorophenol in mixtures.* The total concentration of *meta* or *para* isomer was obtained by measurement of the u.v. absorption in the Unicam SP. 500 spectrophotometer in 0·1 N-NaOH at 299 mμ, where the two isomers have the same extinction coefficient (ε 2400), and by reference to a calibration curve prepared from either *m*- or *p*-chlorophenol. The sum of the concentrations of *meta* and *para* isomers in mixtures was recovered by this method within ±2 % after passage through a partition column.

m-*Chlorophenol in the presence of* p-*chlorophenol.* A suitable volume, usually 0·5–2·0 ml. of the solution of *m*- and *p*-chlorophenol in 0·1 N-NaOH, was neutralized with 0·1 N-HCl and diluted to 10 ml. with water. Saturated aq. $NaHCO_3$ (1 ml.) and aq. 0·1 % Brentamine Fast Red (a stabilized diazotized 5-nitro-*o*-anisidine) were added and the red colour was measured at 500 mμ against a reagent blank after 15 min. in the Unicam SP. 500 spectrophotometer. The reagent was unstable in solution and was prepared immediately before use, but the red colour was stable, when formed, for at least 1 hr. *p*-Chlorophenol also reacted with this reagent but the intensity of the colour was less than 2 % of that given by the *meta* isomer and the concentration of *p*-chlorophenol in mixtures was satisfactorily determined by difference between the sum of *meta* and *para* isomers estimated as above and the colorimetric estimations of *m*-chlorophenol. Recoveries of the individual phenols in mixtures depended on the relative amounts present, but if the ratio of *para* isomer:*meta* isomer was less than 5:1 recoveries of either were within ±10 % after passage through the chromatographic column and extraction from the eluate.

Extraction of monochlorophenols from excreta

Vertebrate urine, containing the equivalent of 0·5 g. of chlorobenzene, was diluted to 200 ml. with water and made 4 N with H_2SO_4. Locust excreta were suspended in 4 N-

H_2SO_4. After refluxing for 4 hr. the volatile phenols were steam-distilled until 500 ml. of distillate was collected. This was made alkaline with a few drops of 40% (w/v) NaOH and evaporated to a syrup *in vacuo*. The small residue was acidified with a few drops of 20N-H_2SO_4 and the liberated phenols were taken up in 100 ml. of *n*-hexane. A portion of this solution containing 5–10 mg. of mixed phenols was then separated on the partition column as described above.

Chromatography of chlorobenzene excreta

Paper chromatography. (*a*) With radioactive excreta. An ethanolic homogenate of excreta from locusts dosed with [^{14}C]chlorobenzene was chromatographed on Whatman no. 4 paper in solvent-system C. Much of the radioactivity located by radioautography and scanning was associated with unidentified material (Table 2), but no unconjugated chloromonophenols (R_F 1·0) were detected on this chromatogram. When excreta containing 0·03 μc of radioactivity were separated in solvent-system D, bands were detected by radioautography which coincided with the chlorophenylglucosides and the chlorophenylcysteines (Table 3). When a similar sample of excreta containing 0·03 μc was hydrolysed by heating at 100° with 2N-HCl for 6 hr. in a sealed tube before chromatography, no glucoside band was detected but the chloromonophenol band was considerably intensified. It was concluded from these chromatographic experiments that the chloromonophenols were excreted mainly as their *O*-conjugates.

(*b*) With non-radioactive excreta. Locusts (200) were each given 0·5 mg. of non-radioactive chlorobenzene and the excreta were collected for 2 days. The excreta were refluxed with 4N-H_2SO_4 for 3 hr. and the cooled solution was continuously extracted with ether for 6 hr. Paper chromatograms

Table 2. *Paper chromatography of unhydrolysed excreta from locusts dosed with* [^{14}C]*chlorobenzene*

Solvent system C was run until the front had moved 12 in.

R_F	Counts/min. at peak activity of zone	Coincidence with reference compounds
0·1	45	·
0·35	40	·
0·50	620	·
0·60	350	Chlorophenylsulphates
0·72	300	Chlorophenylcysteines
0·89	200	Chlorophenylglucosides, chloropolyphenols, chlorophenylmercapturic acids

Table 3. *Paper chromatography of hydrolysed and unhydrolysed excreta from locusts dosed with* [^{14}C]*chlorobenzene*

Solvent system D was run until the front had moved 12 in. Intensity of spots: + +, strong; +, weak; –, nil.

R_F	Intensity of radioautograph spots: Before hydrolysis	Intensity of radioautograph spots: After hydrolysis	Coincidence with reference compounds
0·58	–	+	·
0·61	+ +	+ +	Chlorophenylcysteines
0·68	+ +	–	Chlorophenylglucosides
0·89	+	+ +	Chlorophenols

Table 4. *Spectrophotometric estimation of chloromonophenols present in hydrolysed urines of animals dosed with chlorobenzene*

	Expt. no.	Percentage of dose as chlorophenols: *o*-	*m*-	*p*-	Ratio (*o*-isomer:*m*-isomer:*p*-isomer)
Locusts	1	2·6	2·0	4·2	1·3:1:2·1
	2*	—	4·4	9·4	—:1:2·1
Rabbit	1	1·1	2·8	4·6	0·4:1:1·6
	2	1·2	2·8	6·1	0·4:1:2·2
Rat	1	1·8	2·9	4·9	0·6:1:1·7
	2	2·5	1·8	3·7	1·4:1:2·1
	3	2·8	3·6	6·0	0·8:1:1·7
Ferret	1	1·2	0·8	5·9	1·5:1:7·4
	2	0·4	0·3	2·7	1·3:1:9·0
Cat	1	1·4	3·1	14·9	0·5:1:4·8
	2	1·6	3·1	19·8	0·5:1:6·4

* Estimated by isotopic dilution.

of the extract in the solvents of Table 1 showed the presence of *o*-, *m*- and *p*-chlorophenol and phenol together with a number of apparently polyphenolic products, among which an intense spot of 4-chlorocatechol and a weak spot of 2-chloroquinol were recognized. Similar results were also obtained by paper chromatography of urines of the vertebrates quoted in Table 4, and in the urine of frogs (*Rana temporaria*) and toads (*Bufo bufo*) dosed with chlorobenzene, except that in experiments with frogs only traces of *o*-chlorophenol were found and 4-chlororesorcinol was present in place of 2-chloroquinol.

Quantitative estimation of chlorophenols in locust excreta. Locusts (200) were each dosed with 0·5 mg. of non-radioactive chlorobenzene and excreta were collected for 2 days. The monophenols were separated after hydrolysis and steam-distillation (see above). Analysis by the partition-column method showed that the hydrolysed excreta contained 1·3 mg. of *o*-, 1·0 mg. of *m*- and 2·1 mg. of *p*-chlorophenol. The separated phenols were chromatographed in solvent A and only *m*- and *p*-chlorophenol or *o*-chlorophenol were found in the appropriate extract. Phenol, which was detectable in ether extracts of the hydrolysed excreta, did not appear in the column extract. Similar estimates of the chloromonophenols were made in the urine of vertebrates after dosing with chlorobenzene (0·25 g./kg.) and the results are summarized in Table 4.

Isotopic-dilution procedures

Excreta were collected for 24 or 48 hr. from locusts dosed with [^{14}C]chlorobenzene and the excreta and the locusts were homogenized separately in ethanol. Isotopic dilutions were carried out on both homogenates as described earlier (Gessner & Smith, 1960). Sufficient homogenate was normally taken to give a count rate of at least 5 counts/min. when a compound representing 1% of the dose was being estimated. Re-isolated derivatives were converted into a second derivative which was shown to have the same equivalent specific activity. All derivatives used in the re-isolation procedures were prepared according to the directions of Huntress (1948) or Huntress & Mulliken (1946). Results of the isotopic-dilution analysis are quoted in Table 5.

p-*Chlorophenol.* About 0·5 g. of *p*-chlorophenol was added to the radioactive homogenate and the mixture refluxed with 50 ml. of 5N-NaOH for 24 hr. After cooling and washing with 2 × 50 ml. of ether, the alkaline solution was shaken with benzoyl chloride and the *p*-chlorophenyl benzoate formed was crystallized from ethanol to constant specific activity and m.p. 87°. The purified compound was diluted with a weighed amount of non-radioactive *p*-chlorophenyl benzoate and 1 g. was refluxed for 3 hr. with 3 ml. of triethylene glycol containing 0·5 g. of KOH. After cooling the solution was acidified with conc. HCl and extracted with 5 × 100 ml. of ether. The ether was washed with $NaHCO_3$ to remove HCl and benzoic acid and the chlorophenol was then extracted into 5 ml. of 2N-NaOH. This solution was treated with *m*-nitrobenzoyl chloride and the *p*-chlorophenyl *m*-nitrobenzoate was recrystallized from ethanol until of constant m.p. (124°) and specific activity, which was equivalent to that of the *p*-chlorophenyl benzoate.

m-*Chlorophenol.* The *m*-chlorophenyl benzoate (m.p. 71°) was prepared and crystallized to constant specific activity as described for the *p*-isomer. The derivative was refluxed for 4 hr. with 10 ml. of 10% NaOH for 4 hr., cooled and the solution of *m*-chlorophenol was converted into its *m*-nitrobenzoyl ester. The *m*-chlorophenyl *m*-nitrobenzoate (m.p. 92°) was purified as described above to constant specific activity equivalent to that of the *m*-chlorophenyl benzoate.

o-*Chlorophenol.* The *o*-chlorophenol was mixed with the radioactive homogenate and refluxed in 5N-NaOH as described above, and the phenol was extracted continuously with ether after acidification with 10N-HCl. After removal of the ether the residue was converted into

Table 5. *Metabolites of* [^{14}C]*chlorobenzene*

Results are expressed as percentages of radioactivity in excreta or remaining in locust. —: Not examined.

Dose/locust (mg.)	0·35			0·05	0·45		0·3	0·6
	Excreta							
	First day	Second day	Locust	Locust	Excreta	Locust	Excreta	Excreta
Chlorobenzene*	6·6	10·0	47·5	—	—	—	13·9	—
Phenol	—	—	—	—	—	—	—	2·4
o-Chlorophenol	—	—	—	3·0	1·0	—	—	6·9
m-Chlorophenol	—	—	—	18·1	11·8	8·4	—	—
p-Chlorophenol	25·3	2·18	5·8	—	25	8·4	—	—
4-Chlorocatechol	21·9	8·7	6·4	10·0	—	—	—	—
p-Chlorophenylcysteine*	14·7	4·8	<1·0	8·9	6·1	—	—	4·6
p-Chlorophenylmercapturic acid*	2·6	<3·0	<1·0	—	2·2	—	—	1·1
m-Chlorophenylcysteine*	—	—	—	—	7·9	—	1·1	2·0
m-Chlorophenylmercapturic acid*	—	—	—	—	—	—	—	0·3
o-Chlorophenylcysteine*	—	—	—	—	—	—	—	1·5
Percentage of dose in excreta or locust	25·0 (excreta)	5·5	20·6 (locust)	50 (locust)	37·6 (excreta)	17·8 (locust)	28·5 (excreta)	30·1 (excreta)

* Calculated from figures quoted by Gessner & Smith (1960).

o-chlorophenyl *m'*-nitrobenzoate, which was crystallized to constant specific activity and m.p. (99°) from ethanol.

4-Chlorocatechol. A portion (0·5 g.) was added to the radioactive sample and the mixture made 15 N with H_2SO_4 and kept at 100° for 30 min. The cooled solution was extracted with 4 × 30 ml. of benzene. This solution was dried ($CaCl_2$) and concentrated until crystallization occurred. The 4-chlorocatechol was recrystallized from benzene and light petroleum (b.p. 90–100) until of constant specific activity and m.p. (91°). The purified 4-chlorocatechol was benzoylated with benzoyl chloride and pyridine and the 4-chlorocatechol dibenzoate (m.p. 96°) crystallized from ethanol and from ether until of constant specific activity, which was equivalent to that of the 4-chlorocatechol.

Phenol. The mixture of phenol and radioactive homogenate was refluxed with 2 N-NaOH for 6 hr. and the phenol was continuously extracted with ether after acidification with 10 N-HCl. After removal of the ether, the residue was dissolved in conc. H_2SO_4 and converted into picric acid as described by Huntress & Mulliken (1946). The picric acid was crystallized to constant specific activity and m.p. (122°) from 2 N-HCl. The picric acid (50 mg.) was treated with an equal weight of *p*-toluidine in 2 ml. of water and boiled. The *p*-toluidine picrate, which formed on cooling, was recrystallized (m.p. 170°) from water and had the same equivalent specific activity as the picric acid.

Hydroxylation of chlorobenzene by the 'model' system

Chlorobenzene was suspended in 500 ml. of water and oxidized by the 'model' ascorbic acid–O_2–ferrous iron system (Udenfriend, Clark, Axelrod & Brodie, 1954), as described by Mead, Smith & Williams (1958). After the droplets of chlorobenzene had disappeared (about 0·5 hr.) the solution was acidified and extracted with ether. The ether was evaporated on the water bath and the residue examined by paper chromatography. Spots corresponding to *o*-, *m*- and *p*-chlorophenol, phenol, 4-chlorocatechol and 2-chloroquinol were detected, but the relative amounts of mono- and poly-phenols varied in different experiments. Chloroquinol was always present in much larger amounts than were 4-chlorocatechol or the monophenols, in the 'model' system than in the urines examined; when the experiment was carried on for longer periods chloroquinol appeared to be the major product, along with much unidentified polyphenolic material.

DISCUSSION

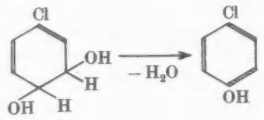
The results obtained show that hydroxylation of chlorobenzene in the locust yields a mixture of phenols similar to that produced by a number of vertebrate species. A large proportion of the phenolic material is 4-chlorocatechol and small amounts of 2-chloroquinol were detected in the excreta of each species. In locust excreta the latter compound was detectable on paper chromatograms from experiments with 200 locusts, but in one attempt to measure it by isotopic dilution it was not present in amounts greater than 1 % of the excreted activity. In addition, the three isomeric chloromonophenols and smaller amounts of phenol were formed by all species examined. The similarity between the phenolic metabolites of chlorobenzene in locusts and vertebrates, especially the presence of *m*- as well as *o*- and *p*-chlorophenol, suggests that similar hydroxylation systems are present in all the species examined. The mixture of phenols found in the excreta was also qualitatively similar to that formed in the 'model' oxidation system, as has been found with other aromatic compounds (Mead *et al.* 1958; Mason, 1957).

Previous observations of the isomer ratios of phenolic metabolites in different species have been confined to compounds which give overwhelmingly *ortho*- or *para*-substituted phenols (Parke & Williams, 1956), and the considerable variation in the *ortho* isomer:*para* isomer ratio found in different animals could have been due, among other possibilities, to steric hindrance or special *ortho*-substitution effects in particular species. Such effects would not operate at the *meta* or *para* positions and, in a compound like chlorobenzene, which gives rise to significant amounts of the *meta*-substituted metabolite, the relative degree of substitution at these two positions should depend on the intimate mechanism of the substitution process *in vivo*. Three mechanisms which have been suggested for this process are the breakdown of premercapturic acids or similar compounds (Knight & Young, 1958; Gessner & Smith, 1960), the dehydration of a dihydrodiol (Boyland, 1950) and a direct hydroxylation following free-radical-substitution rules (Smith, 1950).

It is not thought likely that much of the phenolic material estimated in locust excreta was derived from cysteine-containing precursors since the naphthylpremercapturic acid studied by Knight & Young (1958) and Boyland & Simms (1958) gave only small amounts of naphthols when decomposed in strongly acid solution. The amounts of the isomers of the chlorophenols found in locust excreta were roughly the same as those of various cysteine derivatives present, and paper chromatography suggested that the phenols were excreted as their *O*-conjugates. The presence of *m*-chlorophenol in all the vertebrate urines could be attributed to the decomposition of the premercapturic acid, which gives rise to *p*-chlorophenylmercapturic acid (cf. Gessner & Smith, 1960), since no information is available about the presence or relative amount of any *m*-chlorophenylmercapturic acid in any of the vertebrate species.

The phenols might also be derived from the acid dehydration of a dihydrodiol, such as (I) → (II), or a glucoside conjugate of this (cf. Robinson, Smith & Williams, 1951). The diols do not appear to be biochemical precursors of the phenols in rats since attempts to convert them into phenols in liver

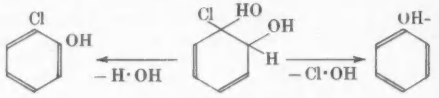
preparations have been unsuccessful (Booth & Boyland, 1957), and it is probable that phenols and dihydrodiols are formed by parallel processes and both are excreted in the urine. The dihydrodiol (I) excreted by the rabbit after dosing with chlorobenzene is known to be converted by acid into *p*-chlorophenol only (Smith *et al.* 1950), and any other possible structure of this type would be expected to give only *o*- or *p*-chlorophenol. The presence therefore of considerable amounts of *m*-chlorophenol in the monophenol fractions from the excreta of all the species examined makes it unlikely that the monophenols are produced solely from the decomposition of dihydrodiols present in the excreta.



(I) (II)

m-Chlorophenol, together with the *ortho* and *para* isomers, is produced from chlorobenzene by chemical hydroxylating systems which probably depend on the generation of free OH radicals, e.g. Fenton's reagent, X-irradiation (Johnson, Stein & Weiss, 1951) and the ascorbic acid–ferrous iron system, so that if the biochemical hydroxylation functions by a free-radical process, *m*-chlorophenol would be an expected metabolite. If this were the case, approximately the same ratio of *o*-, *m*- and *p*-chlorophenol would be expected in each species, provided that it is assumed that the phenols are not further metabolized except by conjugation. When different species were examined it was found that considerable variation occurred in the ratio of the yields of *m*- and *p*-chlorophenol, the two mainly carnivorous species, cat and ferret, excreting considerably more *p*- than *m*-chlorophenol (Table 4). This may be an indication that all the species possess at least two hydroxylation systems, one of which follows the orientation rules of free-radical substitution and gives *o*-, *m*- and *p*-chlorophenol and a second system producing dihydrodiols which, on acid treatment, give the extra *p*-chlorophenol found in the urine. It is also possible that the cat and ferret have an enzyme system capable of converting dihydrodiols into phenols before excretion, since this would also give rise to a relative increase in the amount of *p*-chlorophenol in the urine.

A further point of similarity between the locust and the vertebrate species is the excretion of phenol as a metabolite of chlorobenzene. Chlorine is removed from chlorobenzene by the three chemical hydroxylating systems quoted above and the presence of phenol as a metabolite may be a further indication of an analogous biochemical process. It is equally possible, however, that a 1:2-dihydrodiol (III) is formed, which might be converted either into *o*-chlorophenol or into phenol.



(III)

SUMMARY

1. Phenol, *o*-, *m*- and *p*-chlorophenol and 4-chlorocatechol have been detected in the excreta of locusts dosed with chlorobenzene.

2. The amounts of these phenols present have been determined by isotopic-dilution procedures after dosing locusts with [^{14}C]chlorobenzene.

3. Determination of *o*-, *m*- and *p*-chlorophenol in locust excreta has also been carried out by a chromatographic-colorimetric method and the results have been compared with similar estimations on the urines of cats, ferrets, rats and rabbits dosed with chlorobenzene.

4. The significance of the ratio of *o*-chlorophenol: *m*-chlorophenol: *p*-chlorophenol excreted by different species has been discussed.

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REFERENCES

Azouz, W. M., Parke, D. V. & Williams, R. T. (1952). *Biochem. J.* **52**, 702.
Azouz, W. M., Parke, D. V. & Williams, R. T. (1953). *Biochem. J.* **55**, 146.
Booth, J. & Boyland, E. (1957). *Biochem. J.* **66**, 73.
Boyland, E. (1950). *Symp. biochem. Soc.* **5**, 40.
Boyland, E. & Simms, P. (1958). *Biochem. J.* **68**, 440.
Bradbury, F. R. & Standen, H. (1958). *J. Sci. Fd Agric.* **9**, 203.
Brodie, B. B. & Hogben, C. A. M. (1957). *J. Pharm., Lond.*, **9**, 345.
Burkhardt, G. N. & Lapworth, A. (1926). *J. chem. Soc.* p. 684.
Dennell, R. (1958). *Proc. Roy. Soc.* B, **148**, 280.
Gessner, T. & Smith, J. N. (1960). *Biochem. J.* **75**, 165.
Glazer, E. & Wulwek, W. (1924). *Biochem. Z.* **145**, 514.
Huntress, E. H. (1948). *Organic Chlorine Compounds.* New York: John Wiley and Sons Inc.
Huntress, E. H. & Mulliken, S. P. (1946). *Identification of Pure Organic Compounds.* New York: John Wiley and Sons Inc.
Johnson, G. R. A., Stein, G. & Weiss, J. (1951). *J. chem. Soc.* p. 3275.
Knight, R. H. & Young, L. (1958). *Biochem. J.* **70**, 111.
Mason, H. S. (1957). *Advanc. Enzymol.* **19**, 79.
Mead, J. A. R., Smith, J. N. & Williams, R. T. (1958). *Biochem. J.* **68**, 67.

Milligan, R. F. & Hope, E. J. (1941). *J. Amer. chem. Soc.* **63**, 544.
Mitoma, C., Posner, H. S., Reitz, H. C. & Udenfriend, S. (1956). *Arch. Biochem. Biophys.* **61**, 431.
Parke, D. V. & Williams, R. T. (1956). *Biochem. J.* **63**, 12P.
Peratoner, A. (1898). *Gazz. chim. ital.* **28**, i, 222.
Richards, A. G. (1951). *The Integument of Arthropods.* Minneapolis: University Press.
Robinson, D., Smith, J. N. & Williams, R. T. (1951). *Biochem. J.* **50**, 228.
Smith, J. N. (1950). *Symp. biochem. Soc.* **5**, 15.
Smith, J. N. (1955). *Biol. Rev.* **30**, 455.
Smith, J. N., Smithies, R. H. & Williams, R. T. (1953). *Biochem. J.* **54**, 225.
Smith, J. N., Spencer, B. & Williams, R. T. (1950). *Biochem. J.* **47**, 284.
Terriere, L. C. & Schonbrod, R. D. (1955). *J. econ. Ent.* **48**, 736.
Udenfriend, S., Clark, C. T., Axelrod, J. & Brodie, B. B. (1954). *J. biol. Chem.* **208**, 731.
Wohlleben, W. J. (1909). *Ber. dtsch. chem. Ges.* **42**, 4369.

Biochem. J. (1960) **75**, 179

The Isolation and Characterization of D-Serine Ethanolamine Phosphodiester from Earthworms

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It has been suggested that the biological precursor of lombricine (2-guanidinoethyl 2-amino-2-carboxyethyl hydrogen phosphate, I) is serine ethanolamine phosphodiester (2-aminoethyl 2-amino-2-carboxyethyl hydrogen phosphate, II) and that this may give rise to lombricine by a transamidination reaction involving arginine (Ennor & Morrison, 1958; Rosenberg & Ennor, 1959).

This paper describes a procedure for the isolation of serine ethanolamine phosphodiester, and provides both a complete characterization of the compound and a comparison with synthetic D-serine ethanolamine phosphodiester. The specific rotation of the natural compound was found to be the same as that of the synthetic one, indicating that the serine moiety has the D-configuration. The preparation

$$H_2N{\cdot}C(:NH){\cdot}NH{\cdot}[CH_2]_2{\cdot}O{\cdot}PO(OH){\cdot}O{\cdot}CH_2{\cdot}CH(NH_2){\cdot}CO_2H$$
(I)

$$H_2N{\cdot}[CH_2]_2{\cdot}O{\cdot}PO(OH){\cdot}O{\cdot}CH_2{\cdot}CH(NH_2){\cdot}CO_2H$$
(II)

This suggestion received some support since it was shown that serine ethanolamine phosphodiester did in fact exist in small amounts in earthworm tissue (Rosenberg & Ennor, 1959). The evidence for its presence was based on chromatographic data obtained with the compound itself, its hydrolysis products and its guanylated derivative, for it was not possible, at that time, to obtain it in amounts large enough to permit identification by more conventional means. However, the report of the occurrence of D-serine in lombricine (Beatty, Magrath & Ennor, 1959) suggested that it would be of interest to isolate larger amounts of serine ethanolamine phosphodiester and to determine the configuration of the serine present in this molecule.

procedure has also yielded lombricine in amounts considerably greater than those reported previously (Rosenberg & Ennor, 1959).

MATERIALS AND METHODS

All reagents were analytical grade and solvents used for chromatography were purified by fractional distillation. Synthetic serine ethanolamine phosphodiester (D-SEP) was prepared by Beatty & Magrath (1959) from D-serine.

The earthworms used were as previously described (Rosenberg & Ennor, 1959) and were removed from cold storage ($-70°$) just before use.

Ion-exchange resins and chromatography. The following ion-exchange resins were used: Zeo-Karb 225 (mesh 20–50, H^+ form); Dowex-50 (mesh 200–400, NH_4^+ form); Amberlite CG-120 (mesh 100–200, H^+ form). All resins before use were converted into the H^+ and NH_4^+ forms several times by treatment with 2N-HCl or aq. 2·5N-NH_3 soln. respectively, and when finally in the form required were

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washed with water until the effluent was free from either Cl^- ion or NH_4^+ ion.

Gradient elution. The apparatus used for gradient elution was essentially similar to that described by Hurlbert, Schmitz, Brumm & Potter (1954); the reservoir (5 l.) was initially charged with 4 l. of 1·5 N-perchloric acid and the mixing vessel (2 l.) with 1·5 l. of water. When 2 l. of the mixture had passed through the column the reservoir was replenished with a similar volume of 2 N-perchloric acid and again replenished with 3 N-perchloric acid when a further 3 l. had emerged.

Determination of amino acid. Ninhydrin-reacting components of eluates were determined in a semi-quantitative manner by taking 0·05 ml. of the various fractions and heating at 100° for 5 min. in the presence of one drop of 5 N-HCl, 0·5 ml. of water and 0·5 ml. of 1 % (w/v) ninhydrin in pyridine. When cool the mixtures were diluted to 10 ml. with water and the extinctions measured in a Hilger–Spekker photometer with a spectrum-yellow filter.

Chromatographic solvent systems. Ascending paper chromatography was carried out on Whatman no. 3 papers in ethanol–formic acid–water (70:10:20, by vol.).

RESULTS

Isolation of serine ethanolamine phosphodiester

The extraction procedure was similar to that described for lombricine (Rosenberg & Ennor, 1959), except that the acid extract was not heat-treated. The perchloric acid extract (8 l. from 3·2 kg. of frozen worms) was treated with an equal volume of ethanol and the precipitate (glycogen and fine clay) was removed by centrifuging in a Sharples centrifuge. Potassium hydroxide (10 N) was added to adjust the supernatant to pH 7·0 and the mixture was cooled to 0°. After standing for several hours in the cold room the precipitated $KClO_4$ was removed by filtration and washed with cold 50 % (v/v) ethanol. The combined filtrate and washings (17 l.) were passed through a column (6 cm. × 70 cm.) of Zeo-Karb 225 ion-exchange resin (H^+ form) at the rate of 40 ml./min. The effluent contained a small amount of ninhydrin-reacting material which was found to be free from serine ethanolamine phosphodiester (SEP) and was discarded. The column was then washed with water until the effluent was substantially free from acid (pH 6·0) and the absorbed material eluted with aq. 2·5 N-NH_3 soln. at the rate of 15 ml./min. The eluate was collected automatically in 25 ml. fractions and those containing ninhydrin-reacting material were pooled. The NH_3 was removed under reduced pressure at 40° and the solution (500 ml., pH 7·0–8·0) was applied to a column (78 cm. × 4 cm.) of Amberlite CG-120 ion-exchange resin (H^+ form) at the rate of 20 ml./min. The resin was washed with 200 ml. of water, the column was eluted with perchloric acid by the gradient-elution technique (see Materials and Methods section) at the rate of 5 ml./min. and the eluate collected automatically in 50 ml. fractions. Samples of these were then taken for the colorimetric estimation of ninhydrin-positive material. The elution pattern (Fig. 1) indicated the presence of three major peaks, and samples from the appropriate fractions were taken for paper-chromatographic examination. SEP was found to be present in those fractions at the beginning of peak *B* (Fig. 1) and to be spread over fractions 50–56 (Fig. 2).

The fractions containing SEP were combined, neutralized (pH 7·0) by the addition of 10 N-KOH, cooled to 0° and allowed to stand for 2 hr. The precipitated $KClO_4$ was removed by filtration and washed with 50 ml. of water at 0°. The combined filtrate and washings (500 ml.) were passed through a column (32 cm. × 3·5 cm.) of Zeo-Karb 225 ion-exchange resin (H^+ form, rate 20 ml./min.) and

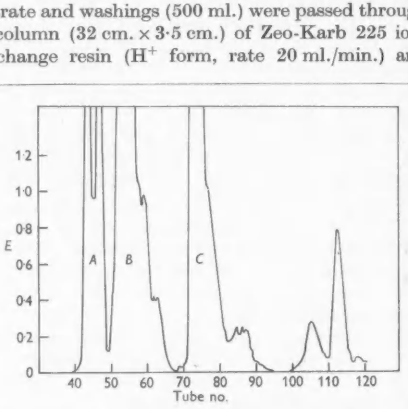

Fig. 1. Elution pattern of ninhydrin-positive material obtained by gradient elution. Details of gradient-elution technique and methods of estimation are given in the text.

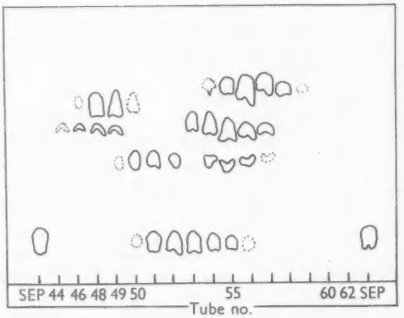

Fig. 2. Ninhydrin-treated paper chromatogram of samples of fractions from peaks *A* and *B* (Fig. 1). Solvent system: ethanol–formic acid–water (70:10:20). Tube nos. were as in Fig. 1; SEP denotes reference standard (synthetic D-SEP). Areas outlined by broken lines indicate faint spots.

eluted as before. Those fractions containing ninhydrin-positive material were combined and evaporated to dryness under reduced pressure at 40°. Water (10 ml.) was added to the dry material (0·9 g.) and a small amount of insoluble residue removed by centrifuging and discarded. The solution was transferred to a column (4 cm. × 20 cm.) of Dowex-50 ion-exchange resin (NH_4^+ form) and allowed to pass through at the rate of 1 ml./min. Water was then passed through the resin at the same rate and the effluent collected in 5 ml. fractions. A sample of each fraction containing ninhydrin-reacting material was taken for paper chromatography, which revealed (Fig. 3) a complete separation of SEP from contaminating ninhydrin-reacting materials. Those fractions containing SEP were combined and evaporated to dryness under reduced pressure at 40°. The dry residue (120 mg.) was combined with the yields of two similar preparations and the combined material (400 mg.) was crystallized from aqueous methanol to yield 0·139 g. of crystalline material. A further yield (0·159 g.) was obtained by successive crystallizations from the mother liquor.

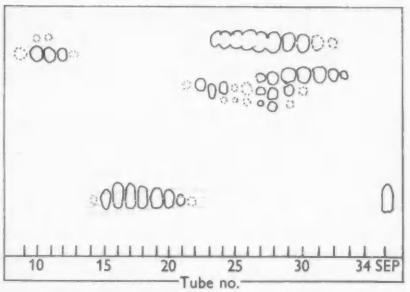

Fig. 3. Ninhydrin-treated paper chromatogram of samples from fractions collected from column of Dowex-50 ion-exchange resin (NH_4^+ form). Solvent system was as in Fig. 2. SEP denotes reference standard (synthetic D-SEP). Areas outlined by broken lines indicate faint spots.

Some physical properties of the isolated SEP are compared with those of synthetic D-SEP. These together with the analytical data are given in Table 1. The infrared spectrum (potassium bromide disk) (Fig. 4) was identical with that of the synthetic SEP and showed peaks at 3430, 2913, 2080, 1682, 1640, 1530, 1462, 1412, 1368, 1345, 1305, 1230, 1075, 1038, 1023, 994, 912, 896, 833 and 779 cm.$^{-1}$.

The results provide proof of the presence of D-serine in SEP.

Isolation of lombricine

Chromatographic examination of the fractions obtained in the gradient-elution step showed the presence of lombricine in peak *C* (Fig. 1) which, between tubes 70 and 83, contained only a minor contamination of ninhydrin-reacting material. The contents of these tubes were combined, neutralized (pH 7·0) by the addition of 10 N-KOH, cooled to 0° and the $KClO_4$ was removed by filtration. The filtrate was freed from $KClO_4$ by passage through Zeo-Karb 225-H and eluted as described above as for the initial extract. The eluate (500 ml.) was reduced in volume to 20 ml. by distillation under reduced pressure and ethanol (3 vol.) was added. Immediate crystallization occurred and, after

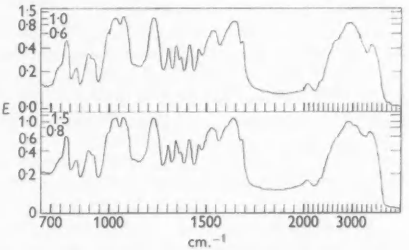

Fig. 4. Infrared spectra. Upper and lower curves represent the spectra of synthetic D-SEP and isolated SEP respectively. Spectra were obtained by the use of a KBr disk in a Perkin–Elmer model 21 double-beam infrared spectrometer.

Table 1. *Analytical data and physical properties of* D-*serine ethanolamine phosphodiester*

		Isolated SEP	Synthetic SEP*	Theoretical for $C_5H_{13}O_6N_2P_{\frac{1}{2}}CH_3{\cdot}OH$
Analyses (%)	C	26·73	—	27·05
	H	6·32	—	6·19
	N	11·17	—	11·48
	P	12·50	—	12·69
M.p. (corr.)		144–145°	144°	—
$\alpha_D^{23\cdot5}$		+18·0°	+18·2°	—
		(*C* = 0·71 in water)	(*C* = 0·66 in water)	—
Crystalline form		Microcrystalline powder, very hygroscopic	—	—

* From Beatty & Magrath (1959).

standing for 2 hr. at 0°, the crystalline material was recovered by filtration, recrystallized from aqueous ethanol and dried *in vacuo* over P_2O_5. Yield was 3·5 g. This material was chromatographically pure and on analysis gave data identical with that previously reported (Rosenberg & Ennor, 1959).

DISCUSSION

Evidence for the presence of serine and ethanolamine in SEP isolated from earthworms was presented previously (Rosenberg & Ennor, 1959).

The present isolation of SEP in amounts sufficient to permit crystallization and chemical and physical analysis has also permitted comparison with D-SEP obtained by synthetic routes involving the use of D-serine. The results leave no doubt about the structure of the isolated material and provide proof of the presence of serine with the D-configuration in the molecule.

The yield of SEP (300 mg. from 9·6 kg. of earthworms), although small, is nevertheless believed to represent the bulk of that present in the starting material, for no loss of ninhydrin-reacting components containing SEP was detected throughout the fractionation procedures. The yield corresponds to 3·1 mg./100 g. and contrasts with the minimal content of 50 mg./100 g. reported by Roberts & Lowe (1954) in river-turtle tissue.

It has been suggested that SEP may be the precursor of lombricine (Ennor & Morrison, 1958; Rosenberg & Ennor, 1959), and the present results indicating the presence of D-serine in SEP (also present in lombricine: Beatty *et al.* 1959) are consistent with this hypothesis.

The procedure developed for the isolation of SEP has yielded lombricine as a 'by-product' in amounts (1·1 g./kg.) appreciably larger than those reported earlier and represents almost a complete recovery of this compound from earthworm tissue.

SUMMARY

1. Serine ethanolamine phosphodiester has been isolated from earthworm tissue in amounts sufficient to permit positive chemical and physical identification.

2. The serine moiety of serine ethanolamine phosphodiester has been shown to possess the D-configuration.

3. The presence of D-serine in the compound is consistent with the thesis that serine ethanolamine phosphodiester is the precursor of lombricine.

4. A simple and improved method for the isolation of lombricine is described.

We are indebted to Dr Joyce Fildes and Dr E. Spinner, Department of Medical Chemistry, for the microchemical and infrared analyses respectively; to Dr D. I. Magrath for advice and to Mr B. Thorpe for skilled technical assistance.

REFERENCES

Beatty, I. M. & Magrath, D. I. (1959). *Nature, Lond.*, **183**, 591.

Beatty, I. M., Magrath, D. I. & Ennor, A. H. (1959). *Nature, Lond.*, **183**, 591.

Ennor, A. H. & Morrison, J. F. (1958). *Physiol. Rev.* **38**, 631.

Hurlbert, R. B., Schmitz, H., Brumm, A. F. & Potter, V. R. (1954). *J. biol. Chem.* **209**, 23.

Roberts, E. & Lowe, I. P. (1954). *J. biol. Chem.* **211**, 1.

Rosenberg, H. & Ennor, A. H. (1959). *Biochem. J.* **73**, 521.

Biochem. J. (1960) **75**, 182

Further Evidence of Tautomerism in Chromatograms of Indolyl-3-Pyruvic Acid

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Indolyl-3-pyruvic acid is a very unstable substance and with the usual methods and solvents cannot be detected in chromatograms, either because it is completely destroyed (Jepson, 1958*a*; Bentley, Farrar, Housley, Smith & Taylor, 1956; Kaper & Veldstra, 1958) or because the solvents used do not separate it from its decomposition products.

With the method of 'double chromatography' (in which the same solvent is used in succession in two directions: Schwarz & Bitancourt, 1957*a*) we have shown that in chromatograms of indolyl-3-pyruvic acid two rows of spots of decomposition products are formed, in the second development, originating from two spots that had been separated in the first development. Both rows reproduce the row of spots of the first development, including the two original spots. We have considered this as evidence of the keto–enol equilibrium of indolyl-3-pyruvic

acid existing in the solution, which is broken during chromatography when the two tautomers are separated (Schwarz & Bitancourt, 1957*b*).

In the present paper it is shown that, with due precautions against oxidation during chromatographic operations, two forms of indolyl-3-pyruvic acid can be demonstrated in chromatograms, as evidenced by the order of appearance of products in solutions of indolyl-3-pyruvic acid decomposed by several means (including changes in pH) and by the colour reactions of the two spots with seven different reagents for the indol, enol and keto groups.

EXPERIMENTAL

The sample of indolyl-3-pyruvic acid was a gift from Dr G. F. Smith (Department of Chemistry, University of Manchester). In the course of our studies it behaved exactly the same as the sample used in previous papers (Schwarz & Bitancourt, 1957*a*, *b*). As explained in those papers this sample was synthesized by Dr A. Ek and, according to the label on the flask received from Dr K .V. Thimann, it is the enol form, with m.p. 211–213°.

A 1% (w/v) stock solution in ethanol was used either directly for the preparation of chromatograms or for preparing more or less diluted aqueous solutions that were decomposed by ageing, heating, u.v. radiation or the pH effect given by sodium bicarbonate. Sodium bicarbonate was dissolved in water that had been boiled for 5 min. and 0·5–1·0 ml. of the 0·1 M solution was added to 1 ml. of ethanolic 0·05 M-indolyl-3-pyruvic acid solution. The stock solution was maintained at −15°; it stayed colourless for at least 5 days, then turned reddish.

Samples of decomposing solutions were drawn at regular intervals so as to have different degrees of decomposition. To avoid decomposition during chromatography, chromatograms were short (10 cm.), which reduced the operation to a duration of about 1 hr. when the solvent was water. As a further precaution against decomposition during chromatography some chromatograms were developed at low temperature in a refrigerator (5–8°) or in a container in which the air had been replaced by nitrogen.

Whatman no. 1 paper was used in ascending chromatography. About 40 μl./spot (4 mm. diam.) was applied at the starting line. The solvents used were: (*a*) water; (*b*) acetone–water (8:2); (*c*) 16% NaCl–2% acetic acid (1:1); (*d*) 25% acetic acid; (*e*) propan-2-ol–aq. 28% NH_3 soln.–water (8:1:1). With (*a*) and (*b*) the chromatogram was usually run in an atmosphere saturated with vapour of acetic acid.

Chromatograms were inspected under long-wave (366 mμ) ultraviolet light (Mineralight lamp SL 3660) and the outline of fluorescent spots was traced with a lead pencil. The outline of non-fluorescent spots which absorb short-wave ultraviolet light and quench the blue fluorescence of the filter paper was traced with a Mineralight SL 2537 lamp (254 mμ).

The following reagents were applied to the chromatogram by spraying, or by application to the chromatogram on a strip of filter paper thoroughly wetted with the reagent, or the spot was cut out from the chromatogram and dipped in the reagent. All of them give sensitive colour reactions with indolyl-3-pyruvic acid but some of them gave better results when applied to the chromatogram while it was still wet, instead of to the dried chromatogram, as is usual.

Gordon–Weber reagent for the indole nucleus (Gordon & Weber, 1951): 0·27 g. of $FeCl_3$ in 100 ml. of 10% perchloric acid. Ehrlich's reagent for the indole and phenol nuclei (Jepson, 1958*b*): 2 g. of *p*-dimethylaminobenzaldehyde, 20 ml. of conc. HCl and 80 ml. of ethanol. 2:6-Dichlorophenolindophenol enol reagent (Stowe, 1955): 2 g. of 2:6-dichlorophenolindophenol in 250 ml. of ethanol and 250 ml. of water. Ferric chloride, used for the quantitative determination of the enol form of acetoacetic ester (Rothe, 1912), was approx. 5% aqueous solution. 2:4-Dinitrophenylhydrazine carbonyl reagent (Jepson, 1958*b*): saturated solution in 2 N-HCl. Van Eck's carbonyl reagent (Linskens, 1955; Melchior, 1957): 5 g. of benzidine, 100 ml. of acetic acid. Ammoniacal $AgNO_3$, used for detection of indolyl-3-pyruvic acid (Bentley *et al.* 1956), enol reagent (Jepson, 1958*b*), 11 g. of $AgNO_3$, 33 ml. of conc. aq. NH_3 soln., 67 ml. of water.

RESULTS

All the following results were confirmed by several repeated experiments.

When the above-mentioned precautions were taken to prevent decomposition during chromatography, fresh solutions of indolyl-3-pyruvic acid chromatographed with water as the solvent in an atmosphere of acetic acid vapour, showed one spot only (spot 2, Fig. 1). This spot has a distinct purplish-blue fluorescence and a faint 'beard' of bluish fluorescence. The upper end of the beard usually exhibits a slightly increased fluorescence and occasionally forms a faint spot (spot 4, Fig. 1). In all the other solvents spot 2 was always accompanied with several spots of decomposition products.

Aerated solutions of indolyl-3-pyruvic acid in sodium bicarbonate decompose rather rapidly, as shown by the quick darkening of the solution. When sodium bicarbonate is dissolved in boiled, air-free water before mixing with the indolyl-3-pyruvic acid solution, darkening is very much delayed and starts at the free surface of the solution, gradually extending to the bottom of the tube. This is especially noticeable when the tube is long and narrow.

In chromatograms prepared in an atmosphere of nitrogen at different time intervals (Fig. 1), from samples drawn from the bottom of the tube, such solutions showed, besides spot 2, spots 3, 4 and 5. Spots 4 and 5 have a bluish and faint-yellowish fluorescence respectively, and spot 3 quenches the blue fluorescence of the filter paper and appears as a dark spot when viewed under short-wave ultraviolet light. In chromatograms of alkaline solutions of indolyl-3-pyruvic acid the maximum relative intensity of spot 2 is obtained when 1 equivalent of sodium bicarbonate is added, that of spot 4 when 2 equivalents are added to the solution.

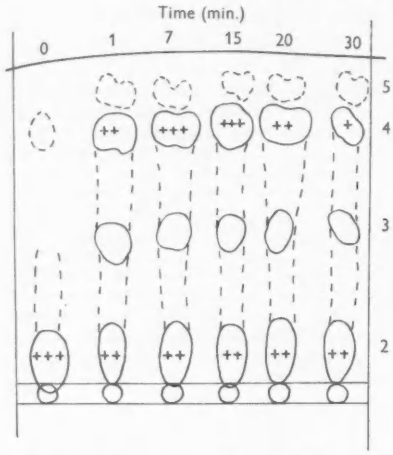

Fig. 1. Paper chromatogram of indolyl-3-pyruvic acid (about 30 μg./spot) from a freshly prepared solution treated with sodium bicarbonate. Samples were drawn at regular intervals during 30 min. of standing at room temperature (20°). Development was in water–acetic acid vapour by the ascending method at 5°. Air in the tank was replaced with nitrogen. The sample at time 0 was drawn from a fresh ethanolic solution of indolyl-3-pyruvic acid. 'Strength' (intensity of fluorescence + size of the spots) of spots 2 and 4 is indicated by the number of crosses. Spots and tails outlined with interrupted lines were very faint.

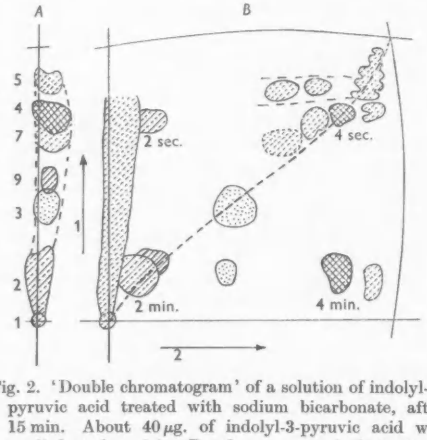

Fig. 2. 'Double chromatogram' of a solution of indolyl-3-pyruvic acid treated with sodium bicarbonate, after 15 min. About 40 μg. of indolyl-3-pyruvic acid was applied at the origin. Development was in both directions in water–acetic acid vapour at 5–8°. Fluorescence colours are indicated at the bottom. *A*, Outline of spots after the first development. *B*, Outline of spots after the second development. Blue; Purple; Red; Yellow; Dark spots.

The fluorescence of spot 2 decreases markedly at first and then remains constant until the end of the experiment (30 min.). Spots 3 and 5 attain maximum intensity within 1 min. and maintain it until almost the end of the experiment. Spot 4 increases until 7 min. and begins to disappear after 30 min.

When air is not excluded from the tank, an additional spot (spot 7, Fig. 2) appears after 30 min. Spot 2 decreases continuously up to the end of the experiment and spot 4, after reaching its maximum, decreases rapidly and practically disappears in 30 min. Spot 5 and the fluorescent tail that bridges all the spots are much more conspicuous.

When the chromatogram is developed a second time with the same solvent in a perpendicular direction ('double chromatograms': Schwarz & Bitancourt, 1957*a*), the same sequence of spots is seen, starting from the sites occupied by spots 2 and 4 in the first development, except that spot 3 is not found in the sequence of spot 4 (Fig. 2*B*).

In chromatograms run with water, only spots 2 and 4 react with all the reagents, both spots responding with the same colorations. Some of these reactions, however, especially those for the enol and for the ketone groups, differ in reaction time and in intensity (Table 1). Whereas the differences are quite conspicuous with some of the reagents when they are applied to the dry chromatograms, in other cases the reagent must be applied while the chromatogram is still wet and as soon as possible after chromatography. With dinitrophenylhydrazine a marked difference in the intensity of reaction of spots 2 and 4 was observed only when the spots were cut from the wet chromatograms and dipped in 2 ml. of the reagent. After 5 min. at 30°, the solution in the tube containing spot 2 was only slightly more yellow than the reagent itself, whereas the colour was brown in spot 4. Both colorations were considerably darker after 1 hr. but kept the same relative intensity. The hydrazones in this reaction, after extraction with ethyl acetate and treatment with a solution of sodium hydroxide, imparted a slight reddish coloration to the aqueous phase with spot 2 and a dark wine colour with spot 4. Whereas spot 3 also gives a strong reaction with dinitrophenylhydrazine, it does not colour the sodium hydroxide solution as would an acid hydrazone (Friedemann & Haugen, 1943).

With dichlorophenolindophenol, which colours the whole filter paper blue or purple, spot 2 decolours it immediately and appears as a white spot. Spot 4 begins to decolour later and takes a few minutes for bleaching. Van Eck's reagent gives a yellow colour immediately with spot 4 and a delayed one with spot 2. Ehrlich's reagent for the indole and phenol nuclei, which gives specific

Table 1. *R_F values and colour reactions of chromatographic spots 2 and 4 (Fig. 1)*

Colour intensity and size of spots are indicated with crosses. Reaction time was instantaneous (inst.) or occurred in minutes.

Chromatography (R_F)	Spot 2	Spot 4
Water	0·07	0·85
Water–acetic acid vapours	0·15	0·80
Water–acetic acid (3:1, v/v)	0·28	?
16% NaCl–2% acetic acid (1:1, v/v)	0·09	0·57
Acetone–water (8:2, v/v)	0·71	0·82
Propan-2-ol–28% ammonia soln.–water (8:1:1, by vol.)	?	0·45*
Enol reagents		
Dichlorophenolindophenol†	+ + + + (inst.)	+ + + (1–2 min.)
Ammoniacal $AgNO_3$‡	+ + + + (inst.)	+ (inst.)
$FeCl_3$‡	+ + + + (inst.)	+ (inst.)
Ketone reagents		
Dinitrophenylhydrazine§	+	+ + +
Dinitrophenylhydrazine + NaOH‖	+	+ + +
Van Eck's reagent†	+ + + + (2–3 min.)	+ + + + (inst.)

* Correspondence with spot 4 not certain.
† On the dry chromatogram. ‡ On the wet chromatogram.
§ Spot cut from wet chromatograms and dipped in 2 ml. of reagent.
‖ NaOH reaction of hydrazone extracted in ethyl acetate from 2 ml. of reagent solution referred to in the preceding footnote (Friedemann & Haugen, 1943).

colours for different indole compounds, gives with spot 2 a yellow colour turning to greenish and with spot 4 a greenish colour which later turns yellow. Ferric chloride and ammoniacal silver nitrate, when applied to the chromatogram while it is still wet, give a strong reaction with spot 2 and a weak one with spot 4.

In chromatograms with propan-2-ol–aq. ammonia soln.–water (8:1:1) the solvent front ascends 10 cm. in 3 hr. and even fresh solutions of indolyl-3-pyruvic acid already show four spots, more or less united by a yellowish fluorescent tail. One of them, with R_F 0·45, is undoubtedly a spot of indolyl-3-pyruvic acid. It shows a bright-blue fluorescence, decolours dichlorophenolindophenol, and gives a purple colour with the Gordon–Weber reagent, pink with ferric chloride, dark brown with silver nitrate, yellow with Van Eck's reagent and brown with dinitrophenylhydrazine. The outline of the colour reactions, however, is not exactly coincidental with that of the fluorescence and extends as tails below and above the outline of the fluorescent spot. Furthermore, when such chromatograms are run in a second direction with water as the solvent, this spot, like spots 2 and 4, shows a row of spots of decomposition products.

The R_F values of spots 2 and 4 in several solvents are given in Table 1. The two spots can easily be identified in all of them, except spot 2 in propan-2-ol and spot 4 in water–acetic acid.

DISCUSSION

Since spot 2 is the only one to appear in chromatograms of fresh solutions, it is undoubtedly a spot of indolylpyruvic acid. This is confirmed by its response to seven different reagents for this substance. After spot 2, spot 4 is the first one to appear in chromatograms of recently prepared solutions. It practically duplicates the properties of spot 2, giving the same colour reactions. In double chromatograms of decomposed indolyl-3-pyruvic acid solutions (Fig. 2), spot 2 as well as spot 4, produced in the first run, give rise, in the second direction, to rows of spots duplicating the sequence of the first run. It seems obvious that spot 2 and spot 4 represent two forms of indolyl-3-pyruvic acid.

As a keto acid, indolyl-3-pyruvic acid may be present in solutions as: (i) ionized and undissociated forms (Bitancourt & Nogueira, 1959); (ii) enol and keto forms; (iii) *cis* and *trans* stereoisomers of the enol form. The possibility that spots 2 and 4 might be the same form in two different states of dissociation seems to be eliminated by the fact that whereas both spots are present in acidic solvents in chromatograms prepared from more or less decomposed solutions, only spot 2 is present in fresh solutions in the same solvent.

Since the sample of indolyl-3-pyruvic acid used in these experiments is the result of chemical synthesis, it may contain the *cis* or the *trans* isomers of the enol form, or both. The fact that there is only one spot of indolyl-3-pyruvic acid in chromatograms from fresh solutions shows either that one of the isomers is present only in very small amounts and is undetectable with our methods, or that it cannot be separated from the other isomer. In either case, spot 4 could not possibly be the *cis* or the *trans* isomer. It should also be noted that

conversion from the *cis* into the *trans* form, or vice versa, can only be made through the opening of the double bond followed by its re-establishment. In other words, there should be at least partial conversion into the keto form and reconversion into the enol form after rotation of the distal end of the side chain. Finally the newly formed stereoisomer should be reconverted into the keto form to give the reactions of spot 4. No evidence of such a succession of conversions appears in our chromatograms.

Several workers have studied the enol–keto equilibrium of indolyl-3-pyruvic acid. Stowe (1955) states that pure indolyl-3-pyruvic acid crystallizes in the enol form and in solution tautomerizes within 20 min. at pH 8·0 and 25° largely to the keto form. Kaper & Veldstra (1958) observed the gradual change of the spectrum of indolyl-3-pyruvic acid in neutral medium and suggested that it was caused by tautomerization of the enol form. As the result of careful u.v.-spectroscopic studies, G. F. Smith (personal communication) has found that 'indolyl-3-pyruvic acid, which in aqueous solution exists almost entirely in the enol form, is converted into β-indolyl-CH_2·CO·CO·O^- on being treated with one equivalent of alkali in the absence of oxygen. If oxygen is not excluded, auto-oxidation occurs. The anaerobic action of the alkali presumably first produces β-indolyl-CH:C(OH)·CO·O^-, which is energetically not favoured, and immediately tautomerizes to the keto form.'

We have found no mention in the literature of the separation of enol–keto tautomers by paper chromatography, but such a separation is to be expected if the tautomers have sufficiently different R_F values and if the reconversion of the two forms, resulting from the re-establishment of equilibrium, proceeds at a slower pace than chromatography. As may be seen in Fig. 1, in the short time that elapses between the addition of sodium bicarbonate to the solution of indolyl-3-pyruvic acid and the preparation of the chromatogram (approx. 1 min.), the substances of spots 3–5 are formed at the expense of spot 2, as indicated by the immediate decrease of its intensity. From then on, during 20 min., little change is observed in the intensity of the spots. This, and the fact that the chromatogram prepared from a fresh solution (on the left of Fig. 1), shows no decomposition products, is a proof that under our experimental conditions there is no oxidation in the chromatogram during chromatography and during the 30 min. it takes to prepare the chromatogram.

Factors that are known to affect the enol–keto equilibrium such as pH, heat, quality of solvent and ageing of solutions (Knorr, Rothe & Averbeck, 1911) produce a decrease of spot 2 and an increase of spot 4. The almost immediate decrease of spot 2 as a result of alkalinization would result both from tautomerization yielding spot 4 and from decomposition yielding spots 3 and 5. The tail of spot 4 indicates the continuance of tautomerization during chromatography. Spot 4 attains its maximum between 7 and 15 min., obviously at the expense of spot 2. As mentioned above, Stowe (1955) observed the conversion of the enol form into the keto form in 20 min. The fact that spot 4 decreases later and is greatly reduced after 30 min. whereas spot 2 stays the same may be due to spot 4 being more readily oxidized than spot 2.

Propan-2-ol–aq. ammonia soln., an alkaline, slow and aggressive solvent, gives in chromatograms of freshly prepared solutions, developed during 3 hr. at room temperature, one spot only of indolyl-3-pyruvic acid (R_F 0·45) as evidenced by six colour reactions. In chromatograms developed with propan-2-ol during 15 hr. indolyl-3-pyruvic acid is completely destroyed (Jepson, 1958*a*, *b*).

The question of which of spots 2 and 4 is the enolic form of indolyl-3-pyruvic acid is of interest. The difference in the colour reactions of these two spots support the conclusion that spot 2 is the enol form and spot 4 the keto form of indolyl-3-pyruvic acid. Both spots give the five reactions for enol and for ketone reagents but in one way or another the enol reactions are stronger with spot 2 and the ketone reactions with spot 4 (Table 1). The reaction is slow for spot 4 with dichlorophenolindophenol, a reagent for the enol form (Stowe, 1955; Linskens, 1955), and the same is true for spot 2 with Van Eck's reagent, specific for the carbonyl group (Linskens, 1955; Melchior, 1957).

Spot 2 responds to Ehrlich's reagent with a yellow colour, changing to green, whereas spot 4 gives a greenish colour, later turning yellowish. It would seem that tautomerization proceeds in spots 2 and 4 during the drying of the filter paper. This may also explain the fact that, with some reagents, differences between spot 2 and 4 are much sharper when the reagent is applied to the chromatogram when it is still wet. The enol reagents ferric chloride and silver nitrate give a much paler reaction with spot 4 when the chromatogram is still wet whereas the reaction is strong with spot 2 whether the chromatogram is wet or dry. Ferric chloride also gives a colour reaction with other indole derivatives but in that case the reaction is much slower than with enolic compounds.

In previous papers it was concluded from spectroscopic considerations (Schwarz, 1957) and from the strong reaction of spot 2 with dinitrophenylhydrazine on the dry chromatogram, in contrast with the faint reaction of spot 4, that spot 2 corresponded to the keto form and spot 4 to the enol form of indolyl-3-pyruvic acid (Schwarz & Bitancourt, 1957*b*). The evidence shown here, however, points to the opposite conclusion. We

have since ascertained that the difference in reaction was mainly due to the very small amounts of the substance of spot 4 produced in our early chromatographic experiments. Furthermore, we have obtained, with dinitrophenylhydrazine, a faint reaction with spot 2 and a strong one with spot 4 by dipping the spots, cut from the chromatograms, in the reagent instead of applying it to the dry chromatogram.

Since spot 2 is the first to appear in chromatograms of freshly prepared solutions the evidence from the colour reactions is that the sample of crystalline indolyl-3-pyruvic acid received from Dr Smith is in the enolic form. In this connexion it is noteworthy that in a recent experiment Dr Smith (personal communication) has obtained an u.v. spectrum corresponding to pure enol from his crystals of indolyl-3-pyruvic acid dissolved in anhydrous peroxide-free dioxan. The preparation proved to be rather stable since E at 328 mμ did not change at all after the first reading, made within 10 sec. of dissolution. Dr Smith thinks this is a reasonable indication that indolyl-3-pyruvic acid must have been in the enol form in the crystalline state.

As mentioned above, Stowe (1955) states that indolyl-3-pyruvic acid crystallizes in the enol form and, from spectrographic studies, Kaper & Veldstra (1958) arrived at the same conclusion. Hydroxyphenylpyruvic acid and phenylpyruvic acid show an analogous behaviour, that is, crystalline acid represents the enol and tautomerizes in aqueous solutions (Knox & Pitt, 1957).

SUMMARY

1. A freshly prepared solution of indolyl-3-pyruvic acid treated with one equivalent of sodium bicarbonate soon begins to decompose. Paper chromatograms of samples drawn at regular intervals show the fading of the original spot (spot 2) and a corresponding appearance of three other spots (3, 4 and 5). The most conspicuous of these, spot 4, first increases and, after 20 min., decreases and fades out.

2. Good separation of spots 2 and 4 is achieved only when precautions are taken against decomposition during chromatography. With a slow solvent like propan-2-ol, in alkaline medium, a number of decomposition products crowd the chromatogram and only a single spot with the characters of spots 2 and 4 can be identified.

3. Colour tests for indolyl-3-pyruvic acid (Gordon–Weber reagent, Ehrlich's reagent, dichlorophenolindophenol, ferric chloride, ammoniacal silver nitrate, dinitrophenylhydrazine and Van Eck's reagent) applied to chromatograms show that only spots 2 and 4 respond to all of them, suggesting that spots 2 and 4 represent two forms of indolyl-3-pyruvic acid.

4. The relative intensity of spots 2 and 4 was shown to depend on pH, temperature, ageing and quality of solvent, i.e. factors influencing tautomerization.

5. It is therefore suggested that spots 2 and 4 correspond to the enol and keto tautomers of indolyl-3-pyruvic acid respectively, and that the tail in between is due to tautomerization during chromatography.

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REFERENCES

Bentley, J. A., Farrer, K. R., Housley, S., Smith, G. F. & Taylor, W. C. (1956). *Biochem. J.* **64**, 44.

Bitancourt, A. A. & Nogueira, A. P. (1959). *Science*, **129**, 99.

Friedemann, T. E. & Haugen, G. E. (1943). *J. biol. Chem.* **147**, 415.

Gordon, S. A. & Weber, H. P. (1951). *Plant Physiol.* **26**, 192.

Jepson, J. B. (1958*a*). *Biochem. J.* **69**, 22P.

Jepson, J. B. (1958*b*). In *Chromatographic Techniques*, p. 114. Ed. by Smith I. London: Heinemann.

Kaper, I. M. & Veldstra, H. (1958). *Biochim. biophys. Acta*, **30**, 401.

Knorr, L., Rothe, O. & Averbeck, H. (1911). *Ber. dtsch. chem. Ges.* **44**, 1138.

Knox, W. E. & Pitt, B. M. (1957). *J. biol. Chem.* **225**, 675.

Linskens, H. F. (1955). *Papierchromatographie in der Botanik*. Berlin: Springer.

Melchior, H. (1957). *Plants*, **50**, 262.

Rothe, O. (1912). Dissertation: University of Jena.

Schwarz, K. (1957). *Arch. Inst. biol.* (*Def. agric. anim.*), *S. Paulo*, **24**, 81.

Schwarz, K. & Bitancourt, A. A. (1957*a*). *Science*, **126**, 607.

Schwarz, K. & Bitancourt, A. A. (1957*b*). *Arch. Inst. biol.* (*Def. agric. anim.*), *S. Paulo*, **24**, 183.

Stowe, B. B. (1955). *Biochem. J.* **61**, ix.

The Differentiation of the A-Type Esterases in Sheep Serum

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The differentiation of paraoxonase (an A-type esterase in serum) from related enzymes has been complicated by overlapping substrate specificities and by the complex nature of these enzymes. Rabbit-serum paraoxonase has been differentiated from hog-kidney di*iso*propyl phosphorofluoridatase by Mounter (1954). He confirmed the observation of Aldridge (1953*b*) that one enzyme in rabbit serum hydrolyses *p*-nitrophenyl acetate and paraoxon (diethyl-*p*-nitrophenyl phosphate) and extended the observation to include tetraethyl pyrophosphate and di*iso*propyl phosphorofluoridate. The hog-kidney enzyme was activated by manganous ions (Mounter & Chanutin, 1953) but did not hydrolyse paraoxon (Mounter, 1956), whereas rabbit-serum paraoxonase was inhibited by manganese and hydrolysed paraoxon. Bergmann, Segal & Rimon (1957) have shown that di-*iso*propyl phosphorofluoridatase does not hydrolyse *p*-nitrophenyl acetate. In addition they have demonstrated a new C-esterase hydrolysing this substance in purified preparations of the hog-kidney enzyme. According to the usage of Aldridge (1954), C-esterase is an A-type esterase. The ability of rabbit-serum paraoxonase to hydrolyse *p*-nitrophenyl acetate could also be used to differentiate paraoxonase and di*iso*propyl phosphorofluoridatase.

The paraoxonases of rabbit, rat and horse sera hydrolysed paraoxon and *p*-nitrophenyl acetate but the ratio of hydrolysis of *p*-nitrophenyl acetate to hydrolysis of paraoxon varied from 4 in rabbit to 18 in horse. From summation experiments Aldridge concluded that one enzyme in these sera was responsible for this hydrolysis and attributed the varying ratios to species differences. However, when the ratios for other sera are calculated, in many cases they are greater. For instance, from Aldridge's results the ratio for ferret serum was 130, for sheep 50, for goat 170 and for human 48. As observed in the present work, the ratio for hog serum was 5700. The possibility that A-type esterases, other than paraoxonase, hydrolysing *p*-nitrophenyl acetate, occur in some sera cannot be excluded. Mounter & Whittaker (1953) suggested that serum arylesterase and paraoxon-hydrolysing A-esterase were identical.

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Although hog-kidney di*iso*propyl phosphorofluoridatase and rabbit-serum paraoxonase can be clearly differentiated, the situation becomes more confused when other tissues, sera and species are examined. Mounter (1955) studied the di*iso*propyl phosphorofluoridatase activities in rat and hog liver and kidney fractions and observed that, unlike the enzyme of the soluble fraction, the enzymes of insoluble fractions of liver were inhibited by manganese and activated by calcium when di-*n*-butyl phosphorofluoridate was the substrate.

The distribution and nature of di*iso*propyl phosphorofluoridatases have been studied in some detail by Mounter, Dien & Chanutin (1955). After comparing these enzymes in the tissues of various species they concluded that a number of di*iso*propyl phosphorofluoridatases exist. Augustinsson & Heimburger (1954) have reported evidence suggesting that serum and kidney tabunase are not identical and also that rabbit-serum tabunase and A-esterase are not identical. On the basis of summation experiments, Cohen & Warringa (1957) were unable to conclude that their highly purified hog-kidney B-fraction, derived from most purified A-2 fraction of Mounter, Floyd & Chanutin (1953), contained only one enzyme hydrolysing di*iso*propyl phosphorofluoridate and tabun (ethyl-*N*-dimethylphosphoramidocyanate).

In the present work a substrate-specificity study with purified paraoxonase preparations from sheep serum (Main, 1960) has been made for the purpose of further differentiating these various activities. Data from other experiments with rabbit, horse, sheep and pig sera have also been employed.

EXPERIMENTAL

Enzyme preparations. Various fractions from the purification of paraoxonase from sheep serum were used. The fractions are designated by their state of purification relative to the starting serum and, when relevant, by the fraction number in the flow scheme employed to illustrate the procedure (Main, 1960). The most pure paraoxonase fractions were purified 305- to 385-fold. Evidence from electrophoresis suggested that the product was 70–85% paraoxonase and contained two components.

Defibrinated sheep, horse, rabbit and rat sera were obtained from recently slaughtered animals and stored in the frozen state.

Enzyme preparations used with most carboxylic ester substrates were first incubated with 1 mM-paraoxon for

30 min. at 37°. For some experiments with phenyl acetate and *p*-nitrophenyl acetate as substrates 10 mM-di*iso*propyl phosphorofluoridate (DFP) was employed. The activity of sheep serum was not affected by previous incubation with paraoxon or DFP.

Estimation of activity. The colorimetric method for paraoxonase previously described (Main, 1960) was frequently employed.

All comparisons of substrate specificity were made with a variation of the manometric method of Aldridge (1953*a*). Generally 0·5 ml. of enzyme preparation was placed in the side arm of the Warburg flask and 2·5 ml. of buffered substrate in the centre well. The final concentrations in the digestion medium, exclusive of substrates, were 0·025 M-$NaHCO_3$, 0·162 M-NaCl and 0·2% of gelatin. The flasks were gassed with CO_2+N_2 (5:95) at room temperature before being placed in a bath at 37°. Manometric readings were made at intervals of 5 min. over a period of at least 20 min. and where possible 30 min. after tipping. In addition, a reading was usually taken 2 or 3 min. after tipping.

The paraoxon K_m of paraoxonase preparations was 14 times higher than that for the sheep serum (Main, 1960). It was therefore desirable to calculate the activity ratios at the maximum velocities where possible, since paraoxon has a limited solubility and the paraoxonase K_m was almost equal to the saturation concentration of paraoxon.

Nitrophenyl esters were prepared by the method of Huggins & Lapides (1947). Stock solutions of the slightly soluble substrates, paraoxon, *o*-, *m*- and *p*-nitrophenyl acetate, *p*-nitrophenyl butyrate and phenyl acetate were prepared by dissolving the ester in methanol or in methanol–acetone (1:1, v/v). Appropriate volumes of the stock solutions were then diluted in buffer to obtain the desired concentration. Appropriate amounts of the more soluble substrates, DFP, tetraethyl pyrophosphate, tabun (ethyl-*N*-dimethylphosphoramidocyanate) and triacetin were dissolved directly in bicarbonate buffer to give a concentration of 12 mM.

Inactivation by heating. About 20 ml. of sheep serum in a thin-walled test tube containing a thermometer was placed in a suitably adjusted constant-temperature bath. Serum samples (2 ml.) were withdrawn at measured time intervals and immediately cooled. Paraoxon hydrolysis was measured colorimetrically with undiluted serum. Determinations of rates of hydrolysis of phenyl acetate, *p*-nitrophenyl acetate and *p*-nitrophenyl butyrate were made by the manometric method with suitably diluted serum.

Inhibition with p-*chloromercuribenzoate.* Sheep serum was diluted with an equal volume of *p*-chloromercuribenzoate solution so that the final concentration of this compound was 0·4 mM. The inhibitor was incubated with the serum for 18 hr. at 4°. The serum hydrolysis rates for paraoxon and *p*-nitrophenyl acetate were then measured manometrically. The manometric buffer initially contained 0·04 M-$MgCl_2$ in addition to the NaCl, $NaHCO_3$ and gelatin. This concentration of magnesium inhibited hydrolysis of paraoxon by 42% and hydrolysis of *p*-nitrophenyl acetate by 65%.

RESULTS AND DISCUSSION

The substrate-specificity pattern of purified paraoxonase preparations is compared with that of the original sheep serum in Table 1. Sheep serum had no measurable B-esterase activity, as defined by Aldridge (1953*a*), towards any of the substrates listed. Previous incubation of serum with 10 mM-DFP or 8 mM-paraoxon for at least 30 min. at 37° did not result in any significant change in the rates of hydrolysis of phenyl acetate, *p*-nitrophenyl acetate and *p*-nitrophenyl butyrate. Sheep serum did not measurably catalyse the hydrolysis of such substrates as triacetin or ethyl butyrate. It was concluded that all the activities compared were of the A-esterase type.

Hydrolysis of p-*nitrophenyl acetate.* The ratio of hydrolysis of *p*-nitrophenyl acetate to hydrolysis of paraoxon at $V_{max.}$ decreased from 67 ± 7 for sheep serum to 9 ± 4 for purified paraoxonase preparation (Table 1). Aldridge (1953*b*) reported that the same ratios in the sera of rabbit, rat and horse were 4, 12 and 18 respectively. From summation experiments he concluded that one enzyme in these sera hydrolysed both *p*-nitrophenyl acetate and paraoxon. The hydrolysis ratios reported by Aldridge are in the same order as those found for the purified paraoxonase preparations examined. However, the sevenfold change in the ratio which resulted from purification suggested the presence in sheep serum of an enzyme, other than paraoxonase, which also hydrolysed *p*-nitrophenyl acetate.

By heat-inactivation and other experiments Mounter (1954) had confirmed the observation of Aldridge (1953*b*) that one enzyme in rabbit serum hydrolysed both *p*-nitrophenyl acetate and paraoxon.

Heat-inactivation studies with sheep serum, as shown in Figs. 1 and 2, presented a different picture. After incubation for 13 hr. at 48°, 31% of *p*-nitrophenyl butyrate-hydrolysing activity and 25% of the *p*-nitrophenyl acetate-hydrolysing activity remained, whereas 97% of the paraoxon-hydrolysing activity had been destroyed. After 19 hr. the paraoxonase activity was not measurable and 10% of the *p*-nitrophenyl acetate-hydrolysing activity and 16% of the *p*-nitrophenyl butyrate-hydrolysing activity remained. These results suggested that one of the enzymes hydrolysing *p*-nitrophenyl carboxylate esters does not hydrolyse paraoxon. This enzyme hydrolysing the acetate and butyrate esters, but not hydrolysing paraoxon, will be called D-esterase to differentiate it from other A-type esterases and from the C-esterase of Bergmann *et al.* (1957). From the results of heat-inactivation experiments of Mounter (1954) D-esterase is evidently absent in rabbit serum. The ratios of *p*-nitrophenyl acetate hydrolysis to paraoxon hydrolysis of sheep (59) and rabbit (4) are consistent with this view.

The ratio of hydrolysis of *p*-nitrophenyl acetate to hydrolysis of *p*-nitrophenyl butyrate changed

significantly from 8 in serum to 14 in preparation C, purified 305 times (Table 1). About 80% of the hydrolysis of *p*-nitrophenyl acetate by sheep serum may evidently be attributed to D-esterase. The ratios at 13 and 19 hr. (Fig. 1) were 6·0 and 5·7 respectively and probably approximated the ratio of D-esterase alone, since the paraoxonase activity at these heating times was negligible. The ratio in rabbit serum was 12 (Aldridge, 1953*a*) compared with 14 in the paraoxonase C preparation. These results suggest that, relative to hydrolysis of *p*-nitrophenyl acetate, D-esterase hydrolyses *p*-nitrophenyl butyrate about twice as readily as paraoxonase. The change in the hydrolysis ratio from 8 in serum to 5·7 in the absence of paraoxonase is consistent with the view that two enzymes in sheep serum hydrolyse *p*-nitrophenyl acetate.

With two purified samples (B and C, Table 1), it was observed that the paraoxon-hydrolysing activity decreased more rapidly during storage than the *p*-nitrophenyl acetate-hydrolysing activity. For example, after 56 days' storage at $-15°$ the paraoxon-hydrolysing activity of sample B decreased from 86 to 56 μl. of carbon dioxide/mg. of protein/min., and the *p*-nitrophenyl acetate-hydrolysing activity had decreased from 1370 ± 37 to 1240 ± 21 μl. of carbon dioxide/mg. of protein/min. Neither the K_m of paraoxon nor the K_m of *p*-nitrophenyl acetate changed significantly during storage. The DFP-hydrolysing activity decreased from 32 to 18 μl. of carbon dioxide/mg. of protein/min., which compared with the loss of paraoxon-hydrolysing activity. As already noted, paraoxonase was more heat-labile than D-esterase. In addition, preliminary results show that the variation in the ratios of *p*-nitrophenyl acetate-hydrolysing to paraoxon-hydrolysing activities of the purified preparations may have resulted from the presence in varying amounts of two imperfectly separated *p*-nitrophenyl acetate-hydrolysing enzymes.

The K_m for hydrolysis of *p*-nitrophenyl acetate and the ratios of its hydrolysis to paraoxon hydrolysis for various samples of enzymes are given in Table 2.

The K_m of purified paraoxonase samples was significantly greater than that in serum and the ratio of hydrolysis of *p*-nitrophenyl acetate to that of paraoxon decreased. Both the K_m and the ratio for the purified paraoxonase samples approached the values for rabbit serum, suggesting a similarity in the paraoxonase of the two sera.

Table 1. *Comparison of substrate specificities*

Activities of three paraoxonase preparations purified from sheep serum are compared with sheep serum. All activities were measured manometrically. Where the K_m values of the substrates compared are known, the activities are compared at their respective $V_{max.}$ as well as at the concentrations given. Paraoxonase preparation A was 385-fold purified, B was 330-fold purified and C was 305-fold purified. The letter after the figure in the purified paraoxonase column refers to the preparation used, as described above. The paraoxon activity was called 1 and the other activities were related to it. The specific activity of the 330-fold purified preparation was 86 μl. of CO_2/mg. of protein/min. at $V_{max.}$ and the activity of the serum from which this preparation was derived was 0·26 μl. of CO_2/mg. of protein/min. Sera used with carboxylic esters were previously incubated with 1 mM-paraoxon for about 30 min. at 37°. Abbreviations used in this table and subsequent tables and figures are: PNPA, *p*-nitrophenyl acetate; MNPA, *m*-nitrophenyl acetate; ONPA, *o*-nitrophenyl acetate; PNPB, *p*-nitrophenyl butyrate; DFP, di*iso*propyl phosphorofluoridate; TEPP, tetraethyl pyrophosphate.

	Comparison at given concn. of substrate		Comparison at $V_{max.}$	
Substrate and concn. (mM)	Sheep serum	Purified paraoxonase	Sheep serum	Purified paraoxonase
Paraoxon (6·7)	1	1	1	1
PNPA (6·7)	68	16 (A)	76	12 (A)
	60	6·5 (B)	68	5 (B)
	49	14·5 (C)	56	11 (C)
DFP (10)	1·05	0·43 (A)	1·83	0·39 (A)
	1·10	0·45 (B)	1·90	0·41 (B)
	1·10	0·45 (C)	1·90	0·41 (C)
MNPA (6·7)	104	28 (C)	—	—
ONPA (6·7)	3·9	1·05 (C)	—	—
PNPB (3·4)	6·3	1·07 (C)	—	—
Phenyl acetate (3·4)	130	0 (C)	—	—
TEPP (10)	0·98	0 (C)	—	—
Tabun (10)	1·36	1·69 (C)	2·44	1·80 (C)
Sarin (10)	0·76	—	—	—
Triacetin (10)	Nil	Nil	—	—

The K_m (0·38 mM) for hydrolysis of *p*-nitrophenyl acetate of the discarded S2 fraction from sheep serum (0·3-fold purified) was 3·7 times less than the serum K_m, and the hydrolysis ratio for *p*-nitrophenyl acetate to paraoxon doubled. The comparable K_m of the A-type esterase in hog serum was 0·485 mM. The A-type esterase in hog serum had a negligible paraoxon-hydrolysing activity (one-fortieth of the activity of sheep serum) but a high *p*-nitrophenyl acetate-hydrolysing activity (three times the activity of sheep serum).

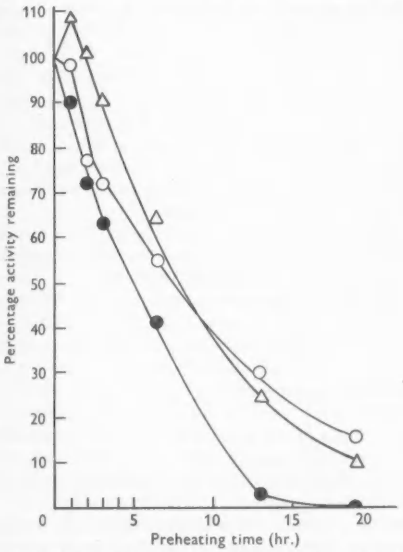

Fig. 1. Effect on the enzymic hydrolysis of *p*-nitrophenyl acetate (PNPA), *p*-nitrophenyl butyrate (PNPB) and paraoxon of preheating sheep serum at 48° for varying times. Percentage of activity remaining: PNPA, △; PNPB, ○; paraoxon, ●. PNPA (6·7 mM) and PNPB (3·4 mM) hydrolysis rates were measured by the manometric method. Paraoxon (8 mM) hydrolysis rates were measured by the colorimetric method.

The *p*-nitrophenyl acetate-hydrolysing enzyme in hog serum appears on the basis of its high activity towards this compound and its K_m to be similar to sheep-serum D-esterase.

This evidence suggested that the K_m for hydrolysis of *p*-nitrophenyl acetate of D-esterase was about 0·4 mM, and of paraoxonase 2 mM, and was consistent with the view that two A-type esterases in serum contribute to the hydrolysis of this compound.

Differentiation of the enzyme hydrolysing phenyl acetate from paraoxonase. Sheep serum hydrolysed phenyl acetate 130 times more rapidly than paraoxon but purified paraoxonase did not hydrolyse it measurably. The amount of paraoxonase used with phenyl acetate was 20 times as great as that used to determine hydrolysis of *p*-nitrophenyl acetate and was twice that used to measure hydrolysis of paraoxon. Nevertheless, the hydrolysis rate remained negligible. The ability to hydrolyse phenyl acetate disappeared in fraction P4 (purified 30 times), suggesting that the phenyl acetate-hydrolysing enzyme may have remained with the cholinesterase in the supernatant S2, which on further precipitation would lead to the equivalent of Cohn fraction IV-4 (Cohn *et al.* 1946).

Fig. 2 shows the effect on the rates of hydrolysis of phenyl acetate, *p*-nitrophenyl acetate and paraoxon of preheating sheep serum for varying lengths of time at 53°. The activity hydrolysing the first

Table 2. *K_m for hydrolysis of* p*-nitrophenyl acetate by various sera and sheep-serum fractions*

K_m values were determined manometrically, typically with four concentrations of *p*-nitrophenyl acetate. The velocities were plotted according to the Dixon (1953) modification of the Lineweaver & Burk (1934) method. Sheep serum (0·02 ml.), 0·02 ml. of rabbit serum, 0·05 ml. of horse serum and 0·01 ml. of hog serum, diluted to 0·5 ml., were placed in the side arm of the manometer flask and 2·5 ml. of buffered solutions of *p*-nitrophenyl acetate in the centre well. Sera used with carboxylic esters were previously incubated for about 30 min. at 37° with paraoxon, usually 1 mM, but with rabbit serum higher concentrations were also used. For explanation of abbreviations see Table 1.

Sample	K_m (mM)	Activity ratio (PNPA/paraoxon) 6·7 mM	$V_{max.}$
Sheep serum	1·42±0·12	6·3	72
Purified paraoxonase (A, B, C; see Table 1)	1·87±0·11	12±5	9±4
Rabbit serum	2·5	4·6	6·3
S2 discarded fraction from purification procedure	0·38	128	—
P2 retained fraction	—	40	44*
Hog serum	0·485	5700	—
Horse serum	1·39	20	—

* Calculated assuming that PNPA K_m was 1·4 mM.

two decreased at a significantly slower rate than that hydrolysing paraoxon. For example, serum heated for 15 min. retained only 29 % of the initial paraoxon-hydrolysing activity but 60 % of phenyl acetate-hydrolysing activity. After heating for 30 min., significant phenyl acetate- and *p*-nitrophenyl acetate-hydrolysing activity remained, whereas the paraoxon-hydrolysing activity had practically disappeared.

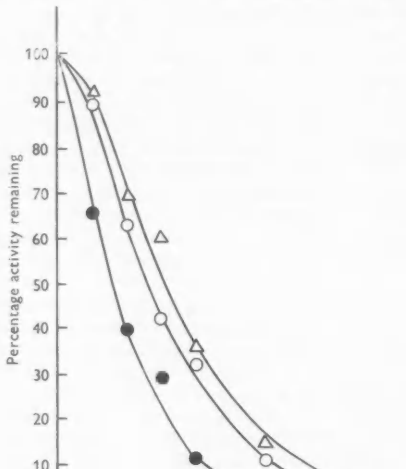

Fig. 2. Effect on the enzymic hydrolysis of *p*-nitrophenyl acetate (PNPA), phenyl acetate and paraoxon of preheating sheep serum at 53° for varying times. Percentage of hydrolysing activity remaining: phenyl acetate, △; PNPA, ○; paraoxon, ●. Phenyl acetate (3·4 mM) and PNPA (6·7 mM) hydrolysis rates were measured by the manometric method. Paraoxon (8 mM) hydrolysis rates were measured by the colorimetric method.

The disappearance of phenyl acetate-hydrolysing activity in a purified paraoxonase preparation, together with the different heat-inactivation rates of paraoxon-hydrolysing and phenyl acetate-hydrolysing activity, suggested that paraoxonase does not hydrolyse phenyl acetate and that the enzyme hydrolysing it does not hydrolyse paraoxon. The suggestion of Mounter & Whittaker (1953) that serum A-esterase, that is paraoxonase, is identical with the enzyme hydrolysing phenyl acetate would not appear on this evidence to be true. The enzyme hydrolysing phenyl acetate must be a distinct A-type arylesterase, since sheep serum does not hydrolyse common ali-esters such as triacetin and ethyl butyrate.

Evidence on the relationship between D-esterase and the enzyme hydrolysing phenyl acetate. The similar heat-inactivation curves for hydrolysis of phenyl acetate and of *p*-nitrophenyl acetate by sheep serum suggested the possibility that one enzyme might be responsible for both hydrolyses after the hydrolysis of the second compound by paraoxonase had been discounted.

The hydrolysis rates of *p*-nitrophenyl acetate, phenyl acetate and paraoxon by the sera of rabbit, sheep, rat and hog are given in Table 3. If D-esterase was responsible for hydrolysis of phenyl acetate then one would expect a high rate of hydrolysis of this compound in hog serum. In fact, hog serum displayed the lowest hydrolysis of phenyl acetate of the sera examined. Rabbit serum, which apparently is free of D-esterase, had the highest phenyl acetate-hydrolysing activity and was 28 times that of hog serum. No consistent relationship between the hydrolysis activities of sera of these species for the three compounds could be detected.

The evidence suggesting that paraoxonase preparations may be contaminated with D-esterase is also not consistent with the idea that D-esterase is identical with phenyl acetate-hydrolysing enzyme, since these samples possessed no activity toward phenyl acetate.

Table 3. *Comparison of the hydrolysis rates of phenyl acetate,* p-*nitrophenyl acetate and paraoxon by the sera of four species*

Activities were determined by the manometric method. Substrate concentrations were: phenyl acetate, 5 mM; PNPA, 6·7 mM; paraoxon, 6·7 mM. Sera used with carboxylic esters were previously incubated for about 30 min. at 37° with paraoxon, usually 1 mM, but in a few instances (phenyl acetate, PNPA) higher concentrations were also used. For explanation of abbreviations see Table 1.

	Activity (μl. of CO_2/ml./min.)			Ratios of activities		
Serum	Paraoxon	Phenyl acetate	PNPA	Phenyl acetate / PNPA	Phenyl acetate / Paraoxon	PNPA / Paraoxon
Rabbit	148	4810	610	7·9	33	4
Sheep	15	2605	770	3·4	173	51
Rat	8	498	92	5·4	62	11
Hog	0·5	170	2830	0·06	340	5660

Table 4. *Effect of* Mn^{2+} *ions on the activity hydrolysing diisopropyl phosphorofluoridate, paraoxon and* p*-nitrophenyl acetate*

1 mM-Mn^{2+} ion was incubated for 20 min. with the enzyme preparation in the side arm of a manometric flask before tipping. For explanation of abbreviations see Table 1. +, Percentage activation; −, percentage inhibition.

	Effect of Mn^{2+} ion on activity (%)				
Substrate and concn. (mM)	Sheep serum	P4 30-fold purified	Paraoxonase 385-fold purified	Horse serum	Rabbit serum
DFP (10)	+40	+14	−87	+42	−14*
Paraoxon (6·7)	−49	−60	−65	−34	—
PNPA (6·7)	−43	−73	−40	—	—

* From Mounter (1954).

The impression gained from this evidence is that D-esterase and the enzyme hydrolysing phenyl acetate are different. The phenyl acetate-hydrolysing A-type esterase may be the arylesterase of Mounter & Whittaker (1953).

Comparison of the hydrolysis rates of p-, m- *and* o*-nitrophenyl acetates.* It was of interest to determine whether the activity of *p*-nitrophenyl acetate-hydrolysing enzymes was dependent on the *para* orientation of the nitro group or whether esters with *ortho* and *meta* nitro groups could also be hydrolysed. For this purpose *o*- and *m*-nitrophenyl acetate, synthesized according to the method used for the *para* compound (Huggins & Lapides, 1947), were used as substrates. The results given in Table 1 show that the *meta* isomer is hydrolysed 2·14 times as fast as the *para* isomer with sheep serum, and 1·83 times as fast with a preparation purified 305 times. The *ortho* isomer was hydrolysed at about one-tenth of the rate for the *para* isomer. The lower hydrolysis rate of the *ortho* isomer may be due to the presence of a blocking ring structure between the adjacent acetate and nitro groups.

The non-enzymic blanks for the *meta* isomer indicated that this ester hydrolysed spontaneously at about half the rate of the *para* isomer. The greater enzymic hydrolysis rate of the *meta* isomer compared with that of the *para* isomer cannot therefore be attributed to the instability of the *meta* isomer. The nitrophenyl acetate-hydrolysing enzymes in sheep serum showed a decreasing activity in the order *meta*, *para*, *ortho*, with respect to the position of the nitro group on the benzene ring. The results also suggest that this may be true to the same extent for paraoxonase, but a definitive statement cannot be made until the possible contamination of the purified paraoxonase with D-esterase is re-examined.

Relationship between C- and D-esterases. Bergmann *et al.* (1957) reported a new C-esterase in the most pure (A-2) di*iso*propyl phosphorofluoridatase fraction of Mounter *et al.* (1953). C-Esterase was not inhibited by DFP, hydrolysed carboxylic esters such as diacetin, *n*-propyl acetate and *p*-nitrophenyl acetate and was relatively resistant to organo mercurials, such as *p*-chloromercuribenzoate. The *p*-chloromercuribenzoate pI_{50} of C-esterase was 2·82.

The *p*-nitrophenyl acetate-hydrolysing activity of sheep serum was inhibited 53% by 0·4 mM-*p*-chloromercuribenzoate, a concentration which activated C-esterase. The paraoxon-hydrolysing activity of sheep serum was inhibited 27% by the compound.

Paraoxonase contributed to the hydrolysis of *p*-nitrophenyl acetate by sheep serum, but since it was less inhibited than the overall *p*-nitrophenyl acetate-hydrolysing activity, the major portion of the inhibited hydrolysing activity must have been associated with D-esterase. The *p*-nitrophenyl acetate-hydrolysing activity of hog serum was inhibited 60% by 0·4 mM-*p*-chloromercuribenzoate. D-Esterase appears therefore to be significantly more sensitive to inhibition by this compound than does either C-esterase or paraoxonase.

Hydrolysis of ethyl butyrate and triacetin by sheep serum was negligible. C- and D-esterase thus appear to differ in their ability to hydrolyse short-chain aliphatic esters, although both hydrolyse *p*-nitrophenyl acetate. This preliminary evidence suggested that C- and D-esterase are not identical.

Evidence for the presence of diisopropyl phosphorofluoridatase in sheep and other serum. This enzyme of hog-kidney purified fractions was activated by manganese (Mounter & Chanutin, 1953, 1954). Similar activation has been observed in other species and tissues, but not in serum. The DFP-hydrolysing activity of rabbit serum (Mounter, 1954) and of certain bacteria (Mounter, Baxter & Chanutin, 1955) was inhibited by manganese.

The effect of manganese on the hydrolysis of DFP, paraoxon and *p*-nitrophenyl acetate is shown in Table 4. With sheep and horse serum, the DFP-hydrolysing activity was activated by manganese, in contrast to its inhibiting effect on rabbit serum. The manganese had little effect unless first incubated

with the enzyme. Manganese concentrations in excess of 1 mM lowered the activation. Mounter & Chanutin (1954) reported that αα-dipyridyl increased the activation effect of manganese with hog-kidney di*iso*propyl phosphorofluoridatase. The addition of αα-dipyridyl almost doubled the manganese activation in sheep serum. The DFP-hydrolysing activity of a paraoxonase preparation purified 385 times was strongly inhibited by manganese. The DFP-hydrolysing activity relative to paraoxon hydrolysis of this and other highly purified fractions decreased by 80 % compared with serum (Table 1). The relative disappearance of DFP-hydrolysing activity in the paraoxonase preparations, together with the change in the effect of manganese on the activity, suggested that sheep serum contained an enzyme similar to the hog-kidney enzyme and that the purification procedure (Main, 1960) separated this di*iso*propyl phosphorofluoridatase from paraoxonase. The observation that the residual DFP-hydrolysing activity in the paraoxonase preparations was inhibited strongly by manganese suggested that this activity was not associated with di*iso*propyl phosphorofluoridatase, but with paraoxonase.

The observation that manganese also activated the DFP-hydrolysing activity of horse serum suggested the possibility that di*iso*propyl phosphorofluoridatase may occur, with paraoxonase, in other mammalian sera.

Aldridge (1953*b*) has observed that other tissues besides sera exhibit paraoxonase activity. The complex effect of manganese on the tissues of various species (Mounter, Dien & Chanutin, 1955) and in hog- and rat-liver fractions (Mounter, 1955) may be partly explained by the presence of varying amounts of di*iso*propyl phosphorofluoridatase and DFP-hydrolysing paraoxonase in these tissues.

Loss of tetraethyl pyrophosphate-hydrolysing activity. The inability of the purified paraoxonase preparations to hydrolyse tetraethyl pyrophosphate suggested that the di*iso*propyl phosphorofluoridatase in the starting sheep serum may have been entirely responsible for this hydrolysis. Mounter & Dien (1956) have reported that purified hog-kidney di*iso*propyl phosphorofluoridatase hydrolysed the compound. At the substrate concentrations used, the hydrolysis rate of tetraethyl pyrophosphate was 80 % of that of DFP. Mounter (1954) reported that one enzyme (paraoxonase) in rabbit serum hydrolysed tetraethyl pyrophosphate, DFP and paraoxon. The hydrolysis rates of tetraethyl pyrophosphate and DFP were comparable and about one-third of the paraoxon-hydrolysis rate. Mounter (1954) based his conclusions on heat-inactivation, heavy-metal-inhibition and summation experiments. However, the preponderance of paraoxonase in rabbit serum may have masked the other enzyme present.

Moreover, some of the criteria used may not have been suitable for differentiating these two enzymes. For example, the rates of inactivation by heating appear to be almost identical for hog-kidney di*iso*propyl phosphorofluoridatase (Mounter & Dien, 1956) and paraoxonase (Main, 1960). Heating for 5 min. at each temperature gave the following results: at 45°, 15 % of the former enzyme and 20 % of paraoxonase were inactivated; at 55°, 95 % and 98 % respectively were inactivated. Both enzymes appear to be inhibited by the same metal ions, with the notable exceptions of magnesium, manganese and cobalt. With most metal ions, similar concentrations give comparable inhibitions, especially when the difficulty of obtaining strictly comparable protein solutions is considered.

The use of summation experiments with two enzymes having overlapping substrate specificities must be regarded with caution, particularly when one enzyme appears to predominate. Tetraethyl pyrophosphate could conceivably act as a competitive inhibitor of paraoxonase. This point calls for further investigation. Although it is not possible at this time to accept without reservation the suggestion that rabbit-serum paraoxonase does not hydrolyse tetraethyl pyrophosphate, nor is it possible to accept the opposite conclusion.

Hydrolysis of tabun. From results on the rates of hydrolysis at $V_{max.}$ sheep-serum paraoxonase appeared to be responsible for about 74 % of the hydrolysis of tabun by sheep serum (Table 1). Cohen & Warringa (1957) reported that a di*iso*propyl phosphorofluoridatase preparation purified from hog kidney hydrolysed tabun and DFP. However, they were unable to conclude that the same enzyme hydrolysed both substrates. It is probable that the di*iso*propyl phosphorofluoridatase in sheep serum is responsible for a portion of the tabun hydrolysis.

SUMMARY

1. The substrate-specificity pattern of paraoxonase preparations purified approximately 300 times has been compared with that of sheep serum.

2. Paraoxonase did not hydrolyse phenyl acetate, whereas sheep serum hydrolysed the compound at a high rate.

3. The high phenyl acetate-hydrolysing activity of sheep serum and the results of heat-inactivation experiments suggested the presence of an A-type arylesterase in sheep serum distinct from paraoxonase.

4. The ratio of *p*-nitrophenyl acetate hydrolysis to paraoxon hydrolysis decreased from 67 ± 7 in serum to 9 ± 4 in purified preparations. Heat in-

activated the *p*-nitrophenyl acetate- and *p*-nitrophenyl butyrate-hydrolysing activity of sheep serum at a significantly slower rate than it did the paraoxon-hydrolysing activity. From this and supporting evidence involving the K_m for hydrolysis activity of sheep, rabbit and hog sera and of purified paraoxonase towards *p*-nitrophenyl acetate, it was concluded that a new esterase hydrolysing *p*-nitrophenyl acetate, but not paraoxon, existed in sheep and probably in hog serum. The esterase was called D-esterase.

5. Evidence was presented which suggested that D-esterase was not identical with the phenyl acetate-hydrolysing arylesterase or with the C-esterase of hog kidney.

6. The effect of manganese and the decrease in the di*iso*propyl phosphorofluoridate-hydrolysing to paraoxon-hydrolysing rate from 1·8 in serum to 0·4 in purified paraoxonase preparations suggested the presence in sheep serum of a di*iso*propyl phosphorofluoridatase similar to that of hog kidney, in addition to paraoxonase.

7. The paraoxonase in sheep serum was responsible for most of the tabun hydrolysis.

8. Sheep-serum and purified paraoxonase preparations hydrolysed *m*-nitrophenyl acetate more rapidly than they hydrolysed *p*-nitrophenyl acetate, and *o*-nitrophenyl acetate was hydrolysed least rapidly.

9. Purified paraoxonase did not hydrolyse tetraethyl pyrophosphate.

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REFERENCES

Aldridge, W. N. (1953*a*). *Biochem. J.* **53**, 111.
Aldridge, W. N. (1953*b*). *Biochem. J.* **53**, 117.
Aldridge, W. N. (1954). *Biochem. J.* **57**, 692.
Augustinsson, K. B. & Heimburger, G. (1954). *Acta chem. scand.* **8**, 1533.
Bergmann, F., Segal, R. & Rimon, S. (1957). *Biochem. J.* **67**, 481.
Cohen, J. A. & Warringa, M. G. P. J. (1957). *Biochim. biophys. Acta*, **26**, 29.
Cohn, E. J., Strong, L. E., Hughes, W. L., Mulford, D. J., Ashworth, J. N., Melin, M. & Taylor, H. L. (1946). *J. Amer. chem. Soc.* **68**, 459.
Dixon, M. (1953). *Biochem. J.* **55**, 170.
Huggins, C. & Lapides, J. (1947). *J. biol. Chem.* **170**, 467.
Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
Main, A. R. (1960). *Biochem. J.* **74**, 10.
Mounter, L. A. (1954). *J. biol. Chem.* **209**, 813.
Mounter, L. A. (1955). *J. biol. Chem.* **215**, 705.
Mounter, L. A. (1956). *J. biol. Chem.* **219**, 677.
Mounter, L. A., Baxter, R. F. & Chanutin, A. (1955). *J. biol. Chem.* **215**, 699.
Mounter, L. A. & Chanutin, A. (1953). *J. biol. Chem.* **204**, 837.
Mounter, L. A. & Chanutin, A. (1954). *J. biol. Chem.* **210**, 219.
Mounter, L. A. & Dien, L. T. H. (1956). *J. biol. Chem.* **219**, 685.
Mounter, L. A., Dien, L. T. H. & Chanutin, A. (1955). *J. biol. Chem.* **215**, 691.
Mounter, L. A., Floyd, C. S. & Chanutin, A. (1953). *J. biol. Chem.* **204**, 221.
Mounter, L. A. & Whittaker, V. P. (1953). *Biochem. J.* **54**, 551.

Biochem. J. (1960) **75**, 195

Methanol Lignin from *Eucalyptus regnans* F. Muell. and its Purification by Countercurrent Distribution

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It is not possible to define lignin satisfactorily from a chemical viewpoint. Brauns (1952) considers it to be a collective term for a group of high-molecular-weight amorphous compounds rather than a constitutionally defined compound. One of the major problems of lignin chemistry is that no simple criterion of purity of a lignin preparation is available.

In view of the above, differences between lignins from different sources and obtained by different methods of preparation are to be expected. On the other hand, agreement between results on one lignin should be attainable if all substances extraneous to the lignin group were eliminated. The first step towards completing and reconciling apparently conflicting results ought to be the preparation of the substance in question in a satisfactory state of purity. The difficulties besetting the worker in the field of lignin chemistry are such that some compromise usually has to be made. It

was shown by Bland (1958*a*) that paper-chromatographic analysis of lignin extracted from *Eucalyptus regnans* with methanol at 150° disclosed the presence of numerous substances, some of them recognizable as lignin-decomposition products. Chromatography showed that repeated precipitation did not free this lignin from all impurities.

The possible advantages of countercurrent distribution as a method for the purification of lignin make its investigation a matter of considerable importance, although development of a suitable solvent system for the resolution of lignin presents some difficulty. Lignin is insoluble in water and therefore this solvent cannot be employed.

This paper describes solvent systems for the purification by countercurrent distribution of lignin extracted from *Eucalyptus regnans* wood by means of methanol at 150° without the addition of acid catalyst. This lignin is referred to as methanol lignin as it is now believed to be similar to that prepared by treatment of wood with methanol–hydrochloric acid. The composition and some of the properties and reactions of the resultant lignin are described.

EXPERIMENTAL

Materials

The methanol-lignin preparations used in these experiments were extracted from different specimens of *E. regnans* wood. One was from immature wood, the trees being about 12 years old, the other from a composite sample of *E. regnans* mature wood. The wood specimens were ground to pass the 1 mm. screen in a Wiley mill, treated with water at about 55° for 8 hr. on three successive days and then treated in the same way with ethanol.

After thorough drying in air, 1 kg. of the wood meal was heated with methanol (10 l.) at 150° for 4 hr., after which the methanol extract was drained off and a further quantity of methanol added and heating continued for 20 hr. The process was repeated with a third quantity of methanol and a 32 hr. heating period. The combined methanol extracts were concentrated to 2·5 l. and poured with stirring into 25 l. of water at 10°. The precipitated lignin was washed and recovered by decantation and centrifuging. Yield was about 50 g.

Countercurrent purification of methanol lignin

Tests with solvent systems showed that possibly useful ones were mixtures of a hydrocarbon or ether with a hydroxylic solvent which separated into two layers on the addition of small amounts of water. The resolution obtained in preliminary runs was followed with the aid of paper chromatography, specimens from the emergent upper layers and from the tubes of the Craig machine being concentrated in groups. The concentrates were spotted on to Whatman no. 1 paper which had been dipped in 0·05 M-sodium tetraborate solution and air-dried. The sheet was irrigated for 6 hr. by the ascending technique with *iso*butanol–benzene–water (1:9:10), as used by Bland & Gatley (1954). Efficiency of the countercurrent separation was then roughly assessed by visual inspection of the sheets in ultra-violet light. The two solvents selected as promising were: (1) benzene–methanol–water (200:100:36, by vol.); (2) methanol–*iso*propyl ether–water (2:4:1, by vol.). The lower layer in each system was rich in methanol; in solvent 1, decreasing the proportion of water from 36 to 30 at 20° gave a methanol-rich upper layer.

Progress of the separation was assessed quantitatively by cutting out the visible spots and determining the amounts present by eluting from the paper and measuring the extinction of the eluate. This procedure was similar to that employed by Bland & Stamp (1955) in the determination of oxidation products of lignin by chromatography. Two substances not completely separated by the countercurrent-distribution procedure but separated by paper chromatography could thus be traced and their overlapping distribution curves accurately plotted. When the separation had proceeded to a stage where the chromatogram indicated that the main lignin fraction had been freed from degraded lignin and other materials the emergent upper layers and the contents of the tubes of the Craig machine were grouped and concentrated. The lignin was recovered by precipitation into cold ether from the concentrate of the appropriate tubes. After purification on the Craig machine the main lignin fraction was subjected to further paper-chromatographic examination with benzene containing different percentages of *N*-dimethylformamide. The degraded lignin, which passed into the emergent upper layers, was recovered by extraction of the grouped and concentrated upper layers with aqueous sodium hydroxide followed by precipitation with acid.

Examination of purified lignins

Sulphuric acid treatment. A specimen (0·2 g.) of the lignin was stirred at 20° for 2 hr. with 72% sulphuric acid (15 ml.), which was then diluted to 3% and boiled for 2 hr. The lignin was recovered by filtration on an alundum crucible, washing with hot water and drying at 105°. The resultant lignin was analysed for C, H, O and O·CH_3.

Infrared spectra. The infrared-absorption spectra were determined in potassium chloride disks as described by Higgins (1957).

Determination of phenolic groups. Phenolic groups were determined by a modification of the method of Butler & Czepiel (1956), which involves potentiometric titration of lignin with sodium hydroxide in methanol with *N*-dimethylformamide as solvent. The solvent was titrated to the point where the observed e.m.f. ceased to rise rapidly; a quantity of the lignin sample (about 0·1 g.) was then introduced and the titration continued until the e.m.f. again ceased to rise. A weighed quantity of the lignin (about 0·2 g.) was then introduced and the titration continued until the e.m.f. reached a steady value. A plot of observed e.m.f. against volume (ml.) of alkali added gave a discontinuous curve with two rising parts after each addition of lignin. On each one of these the point of inflexion could be distinguished and the titre of the second quantity of lignin was taken as the difference between the titres at the two points of inflexion (see Fig. 3). This method eliminated uncertainty of the starting point of the titration. Phenolic groups were also determined by the barium chloride method of Enkvist, Alm & Holm (1956) and by the spectrophotometric method of Aulin-Erdtman (1954).

Total hydroxyl content. This was determined by the method of Hillis (1954).

Detection of hydroxybenzyl alcohol group. These were tested for by the quinonemonochloroimide method of Gierer (1954).

Nitrobenzene oxidation. The lignin (0·05 g.) was placed in a stainless-steel bomb (10 ml. capacity) with 2N-sodium hydroxide (5 ml.) and 0·2 ml. of nitrobenzene. The sealed bomb was heated for 3 hr. at 160°, cooled, opened and the contents were acidified to about pH 3·0 with hydrochloric acid and allowed to stand overnight. The mixture was then centrifuged and the residue washed with water (3 × 30 ml.). The supernatant solution and washings were combined and extracted with chloroform (4 × 10 ml.) and the chloroform extracts were evaporated to dryness at room temperature. The dry residue was dissolved in ethanol (2 ml.) and the aldehydes were separated chromatographically on borate paper with methanol–*iso*propyl ether (peroxide-free)–water (1:1:1, by vol.) and determined according to Bland & Stamp (1955).

Spectrophotometric determination of syringyl and guaiacyl units. The molecular extinction coefficient, ϵ, based on the mol.wt. of the C_9 unit of the purified lignins, was determined and an estimate made of the ratio of syringyl to guaiacyl units by comparison with the molecular extinctions of syringyl alcohol and vanillyl alcohol. The $\Delta\epsilon$ ($\epsilon_{alkaline} - \epsilon_{neutral}$) curves for the lignins were determined and an estimate of the number of phenolic units was made by comparison of $\Delta\epsilon_{max.}$ for the lignins with $\Delta\epsilon_{max.}$ for the two alcohols. The same $\Delta\epsilon$ curves were used to obtain an estimate of the proportion of syringyl units in the total phenolic units. The determinations were made in neutral and alkaline (0·1N-potassium hydroxide) methanol with an Optica recording spectrophotometer.

RESULTS

Some difficulty was experienced with the benzene–methanol–water mixture as it tended to produce a coarse emulsion of the upper and lower layers, the separation of which was imperfect even after a settling period of 15 min. Nevertheless, as preliminary experiments showed that it brought about a definite resolution of the crude methanol lignin its use was continued. With this solvent system the partition coefficient of the main lignin fraction was such that it always passed mainly into the methanol-rich phase. When this was the upper (mobile) phase the lignin therefore passed rapidly along the machine into the mergent upper layers, when no further separation was possible. When the methanol-rich phase was the lower (stationary) phase the main lignin fraction remained in the first few tubes of the train and a large number of transfers could be employed. The number of transfers is, however, limited by other factors such as losses by spillage resulting from imperfect separation of the two layers. The results of such a resolution with 200 transfers are shown graphically in Fig. 1, from which it may be seen that the substances of low R_F remained in the first 30 tubes, whereas those of higher R_F were carried forward. Only the substances of low R_F had u.v.-absorption spectra typical of lignin.

The fraction which passed forward in the top layer of the solvent could not be recovered by ether precipitation, since it was soluble in ether. This material was spread over a large range of the emergent upper layers, from which it was recovered by extraction with sodium hydroxide and reprecipitation with acid. This precipitate was obtained almost ash-free after exhaustive washing with water. Paper-chromatographic analysis of this product showed it to consist of materials of a wide range of R_F and not to correspond to any one of the lignins distinguished chromatographically in the original crude lignin. Its ultraviolet-absorption spectrum lacked the typical maximum of lignin in the 275 mμ region. The infrared-absorption spectrum (potassium chloride disk, Fig. 2) may be seen by comparison with the curve of the main lignin

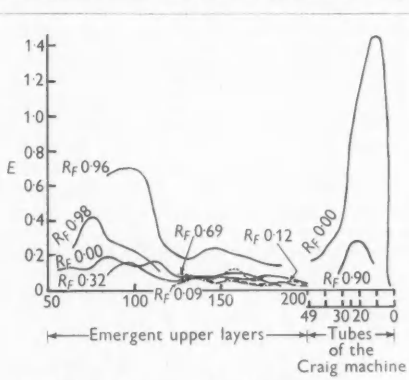

Fig. 1. Distribution curves of crude methanol lignin with benzene–methanol–water solvent system. R_F values were determined with *iso*butanol–benzene–water (1:9:10, by vol.).

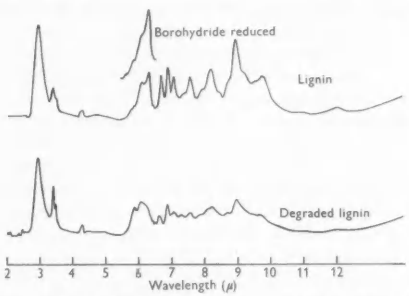

Fig. 2. Infrared-absorption curve of the lignins I–IV showing removal of band at 6 μ by borohydride reduction and absorption curve of degraded lignin.

Table 1. *Composition of lignins from mature and immature* Eucalyptus regnans *wood after purification by countercurrent distribution with different solvents*

Nature of wood	Solvent	Lignin	Absorption maximum (mμ)	Composition (%) C	H	O	O·CH_3	C_9 formula
Immature	Benzene–methanol–water	I	275	58·78	6·21	32·9	26·01	$C_9H_{8\cdot08}O_{2\cdot70}$ $(O\cdot CH_3)_{1\cdot86}$
Immature	*iso*Propyl ether–methanol–water	II	275	59·02	6·26	33·6	26·21	$C_9H_{8\cdot13}O_{2\cdot77}$ $(O\cdot CH_3)_{1\cdot87}$
Mature	Benzene–methanol–water	III	272	58·68	6·25	34·2	25·40	$C_9H_{8\cdot20}O_{2\cdot92}$ $(O\cdot CH_3)_{1\cdot81}$
Mature	*iso*Propyl ether–methanol–water	IV	272	59·11	6·29	33·5	27·11	$C_9H_{8\cdot05}O_{2\cdot71}$ $(O\cdot CH_3)_{1\cdot94}$

Table 2. *Chromatographic resolution of main lignin fraction*

Paper chromatography with dimethylformamide–benzene, of the lignin fractions (Table 1) gave two spots. The relative amount of material in each spot depended upon the composition of the solvent, as shown.

Percentage of dimethylformamide in solvent mixture	Percentage of lignin R_F 0	R_F 0·95
5	83	17
7	69	31
10	23	77
15	16	84

fraction to have the same general shape, but the bands are less well defined. An additional band is present at 5·9 μ and this may correspond to the generation of a carbonyl group, as found by Sarkanen & Schuerch (1957) to take place during alcoholysis of lignin. Unlike the main lignin fraction, this material, which appears to be correctly termed degraded lignin, could not be prepared to a constant composition.

The main lignin fractions, recovered from immature wood with solvents 1 and 2, and from mature wood with solvents 1 and 2, have been numbered I to IV respectively, as shown in Table 1. When recovered by precipitation into ether they were light buff or almost white. They were recovered in a yield of 40–50 % of the original precipitated lignin and chromatographic analysis showed them to be free from the low-molecular-weight material associated with the original lignin. However, resolution on plain paper with benzene–dimethylformamide for 6 hr. gave two fractions as shown in Table 2. It can be seen by reference to this table that when the two lignins were estimated by elution and determination of $E_{275\ m\mu}$ the relative amounts depended on the percentage of dimethylformamide in the solvent mixture. It thus appears that countercurrent distribution gave a lignin of narrow partition-coefficient limits but containing substances of differing dissociation constants. They did not show a definite melting point but shrank at about 130° and softened over a range of roughly 20°. It is clear that the lignins isolated were not pure substances in the chemical sense but were purified lignins in Brauns's sense of lignin as a group of substances. The examination of these lignins is of interest to establish the basic structure of eucalypt lignin and to compare with other lignins.

The analyses presented in Table 1 show no significant difference between lignins recovered with either of the two solvent systems or between lignins from immature or mature wood. There is, however, a difference in the position of the ultraviolet-absorption maximum of lignin from mature and immature wood and this is the same as observed earlier by Bland (1958*b*) for lignin separated by paper chromatography, i.e. the maximum of the immature-wood lignin lies at 275 mμ and that of the mature-wood lignin at a shorter wavelength, here 272 mμ. The infrared-absorption spectra of the four lignins were identical, the curve being given in Fig. 2.

The molecular weights of lignins I and IV determined by the Rast method were 2235 and 2180 respectively. The sulphuric acid treatment of lignin I yielded 90 % of acid lignin [Found C, 60·68; H, 5·80; O, 31·30; ash, 1·57; O·CH_3, 22·37 %; equivalent to $C_9H_{7\cdot46}O_{2\cdot57}$ $(O\cdot CH_3)_{1\cdot65}$]. This corresponds to the loss of 0·41 methoxyl group per C_9 unit during the acid treatment and probably represents the methoxyl group introduced during the methanol extraction.

The results of the determination of hydroxyl groups in lignins I and IV are shown in Table 3. The phenolic hydroxyl-group content of lignin I, determined by the potentiometric method as illustrated in Fig. 3, corresponds to 0·35 hydroxyl group per C_9 unit, whereas the total hydroxyl-group content corresponds to 1·40, giving the aliphatic hydroxyl-group content by difference as

1·05 per C_9 unit. It appears that the C_9 formula of lignin I may be expressed as

$$C_9H_{6\cdot68}O_{1\cdot30}(OH)_{1\cdot05}(OH)_{0\cdot35}(O\cdot CH_3)_{1\cdot86},$$

but this requires further examination.

Treatment of the lignins I–IV with *p*-quinonemonochloroimide according to Gierer (1954) gave no colour, thus showing *p*-hydroxybenzyl alcohol groups to be absent. The original wood gave a positive reaction corresponding to less than 0·01 *p*-hydroxybenzyl alcohol group per phenylpropane unit; this agrees with the conclusion of Adler (1958) about spruce lignin. This indicates that the *p*-hydroxybenzyl alcohol groups were methylated during the extraction but as the proportion of them in the original lignin was very low this reaction accounts only for a very small part of the introduced methoxyl group.

Adler & Gierer (1955) found that in Brauns's native lignin from spruce, treatment with methanol–hydrochloric acid, either at room temperature or under reflux, did not cause the disappearance from the infrared spectrum of the carbonyl-group band at about 1660 cm.$^{-1}$ (6 μ), but reduction with sodium borohydride caused its disappearance. From the spectra of lignin I in Fig. 2 it is clear that this band is intact and shows that the carbonyl groups did not react with methanol at 150° but were removed by reduction with borohydride.

The phenolic hydroxyl-group content of the eucalypt methanol lignin per C_9 unit, 0·35, is similar to that found by Bland (unpublished work) in eucalypt milled-wood lignin (about 0·37) and to that found in spruce milled-wood lignin (0·35) by Enkvist *et al.* (1956). There is no indication therefore of any change in the number of phenolic groups during the methanol extraction. Adler (1958) concluded that in conifer lignin the introduction of 0·42 methoxyl group per original methoxyl group took place at arylcarbinol and arylcarbinol-alkyl ether groups. It would appear, therefore, that the reaction of eucalypt lignin with methanol at 150° was similar to the reaction with methanol–hydrochloric acid at room temperature, and it may be concluded that the methylation took place most probably at arylcarbinol or arylcarbinol-alkyl ether groups. However, the 'total hydroxyl' determined by acetylation probably includes the introduced methoxyl groups, as these were removed by the sulphuric acid treatment and similar ethyl groups were unstable even in dilute acid (Bland, Billek, Gruber & Kratzl, 1959). These results suggest that the composition of the original lignin in the wood may be expressed as:

$$C_9H_{6\cdot68}O_{1\cdot30}(OH)_{1\cdot05}(OH)_{0\cdot35}(O\cdot CH_3)_{1\cdot45}.$$

This C_9 formula may be compared with those derived for spruce lignin by various workers. Erdtman, Lindgren & Petterson (1950), on the assumption that sulphonation occurred by substitution of hydroxyl groups by sulphonic acid groups, deduced the formula

$$C_9H_{6\cdot8}O_{1\cdot0}(OH)_{1\cdot53}(O\cdot CH_3)_{0\cdot94}$$

for protolignin. Arlt, Sarkanen & Schuerch (1956) deduced $C_9H_{7\cdot6}O_{2\cdot6}(O\cdot CH_3)_{0\cdot95}$ from the composition of their ethanol lignin. Björkman & Person (1957) found

$$C_9H_{7\cdot68}O_{1\cdot22}(OH)_{0\cdot30}(OH)_{0\cdot85}(O\cdot CH_3)_{0\cdot96}$$

for milled-wood lignin from *Picea abies* and

$$C_9H_{9\cdot03}O_{2\cdot77}(O\cdot CH_3)_{1\cdot58}$$

for milled-wood lignin from *Betula verrucosa*. Merewether (1954) reached a formula for eucalypt

Table 3. *Hydroxyl-group contents of lignins*

	Phenolic hydroxyl group (%)		Total hydroxyl group (%) (Acetylation)
	Potentiometric	$BaCl_2$	
Lignin I	2·85	2·03	11·27
	2·86	2·26	10·74
	2·58	2·13	—
Lignin IV	2·69	2·51	—
	2·72	2·30	—
	2·84	2·38	—

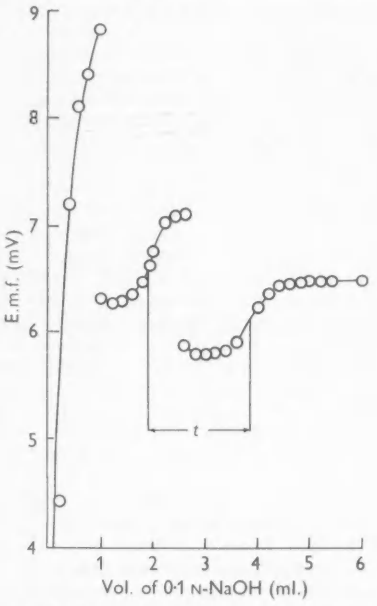

Fig. 3. Specimen curves of potentiometric determination of phenolic groups in lignin (*t*, titre).

Table 4. *Spectrophotometric data on methanol lignins and related compounds*

Substance	Neutral		Difference spectrum ($\Delta\epsilon$)				
	$\lambda_{max.}$ (mμ)	$\epsilon_{max.}$	$\lambda_{max.}$ (mμ)	$\Delta\epsilon_{max.}$	$\lambda_{min.}$ (mμ)	$\Delta\epsilon_{min.}$	$\frac{\Delta\epsilon_{max.}}{9245}$
Eucalypt lignin I	275	2060	258	1 940	282	587	0·21
Eucalypt lignin IV	272	2140	258	1 820	282	482	0·20
P. radiata							
Methanol lignin	280	3100	251	1 985	280	78	—
Vanillyl alcohol	280	2900	253	10 090	278	0	—
Syringyl alcohol	272	1100	260	8 400	283	3858	—

protolignin which, expressed on the same basis, becomes $C_9H_{7\cdot17}O_{2\cdot90}(O{\cdot}CH_3)_{1\cdot37}$ or

$$C_9H_{5\cdot80}O_{1\cdot53}(OH)_{1\cdot37}(O{\cdot}CH_3)_{1\cdot37}.$$

Nitrobenzene oxidation of lignin I gave 26·9 % of syringaldehyde and 5·9 % of vanillin; lignin IV gave 24·3 % of syringaldehyde and 5·8 % of vanillin. These results demonstrate the presence of syringyl and guaiacyl residues but do not disclose their relative numbers in the lignin. Freudenberg & Schlüter (1955) have pointed out that the low yield of vanillin is connected with the linkage of guaiacyl residues by C–C bonds at the position *ortho* to the hydroxyl group whereas this is not possible for the syringyl residues.

An independent estimate of the relative numbers of syringyl and guaiacyl units can be obtained from the spectrophotometric data on lignins. Aulin-Erdtman (1953*a*, 1957) reported that for vanillyl (guaiacyl) alcohol and syringyl alcohol $\log \epsilon_{280} = 3{\cdot}47$ and $\log \epsilon_{271} = 3{\cdot}05$ respectively, and that for spruce methanol lignin $\log \epsilon_{280} = 3{\cdot}5$. The ϵ values for lignins I and IV together with those for *Pinus radiata* methanol lignin and vanillyl alcohol and syringyl alcohol determined under the same conditions are shown in Table 4. It may be seen by reference to this Table that $\epsilon_{max.}$ of lignins I and IV corresponds to the presence of approximately equal numbers of guaiacyl and syringyl units.

The difference values, $\Delta\epsilon_{max.}$, for lignins I and IV shown in Table 4 indicate that 0·2, i.e. one in five, of the C_9 units of these two lignins are phenolic and ionized in methanolic 0·1 N-potassium hydroxide. This is considerably lower than the total phenolic hydroxyl-group content determined by the potentiometric method, which indicated one in three of the C_9 units to be phenolic. The low results given by the $\Delta\epsilon$ method for phenolic content have previously been discussed by Freudenberg & Dall (1955).

The $\Delta\epsilon_{min.}$ at about 280 mμ gives an estimate of the fraction of syringyl units in the total phenolic units ionized in methanolic 0·1 N-potassium hydroxide, as Aulin-Erdtman (1953*b*, 1957) has shown that the $\Delta\epsilon_{min.}$ for vanillyl alcohol is close to zero but for syringyl alcohol $\Delta\epsilon_{min.}$ has a positive value. However, it may be seen from Table 4 that *Pinus radiata* methanol lignin gave a small positive value for $\Delta\epsilon_{min.}$, although Bland, Ho & Cohen (1950) showed that *P. radiata* contained no syringyl groups. This may be due to the influence of conjugated chromophores in the lignin, and as the long-wavelength (200–300 mμ) portion of the $\Delta\epsilon$ curve for *P. radiata* lignin was similar to that of the eucalypt lignins, the $\Delta\epsilon_{min.}$ value of the *P. radiata* lignin was used as a first approximation to correct the $\Delta\epsilon_{min.}$ values of the eucalypt lignins. This led to the conclusion that about 60 % of the ionized phenolic groups were syringyl groups.

DISCUSSION

There was no reason for assuming that countercurrent resolution of a lignin preparation would give one or more clearcut lignin fractions. A continuous distribution of partition coefficients in the lignin would have led to a continuous distribution of lignin along the train, but in fact the main lignin fraction appeared as the usual type of distribution curve. However, it cannot be claimed that countercurrent distribution provides a method whereby lignin can be prepared as a pure substance in the strictly chemical sense. Nor would the methods described necessarily apply to other lignin preparations, especially those of higher molecular weight, such as milled-wood lignin. On the other hand, where applicable as a method of purification it is probably more satisfactory than repeated precipitation as it eliminates adsorption of impurities by the lignin. The 'pure' lignin obtained had properties in accordance with Brauns's conception of lignin as a group of amorphous compounds.

The results of chemical examination of this lignin can be accepted as referring to the lignin itself and not partly to adsorbed degradation products or other impurities. The results of Bland *et al.* (1959) suggest that lignin extracted with methanol at high temperatures is an altered lignin, as when labelled methanol was used not all of the activity could be recovered by Zeisel distillation. However, comparison of results on this eucalypt

lignin with those of other workers on spruce lignin show the eucalypt lignin to have striking resemblance in chemical composition and properties to spruce lignin and suggests that, despite the presence of syringyl groups, the basic structure of the two lignins is fundamentally the same.

SUMMARY

1. Crude methanol lignin from immature and mature *Eucalyptus regnans* has been separated by countercurrent distribution into lignin and degraded lignin.

2. Chromatographic analysis of the separated lignin indicates the presence of a number of substances of differing dissociation constants. This is in accord with Brauns's conception of lignin as a group of amorphous substances.

3. The lignin of immature *E. regnans* is shown to possess the empirical formula based on a C_9 unit of $C_9H_{6\cdot68}O_{1\cdot30}(OH)_{1\cdot05}(OH)_{0\cdot35}(O{\cdot}CH_3)_{1\cdot45}$, where the aliphatic hydroxyl-group content is represented by 1·05 and the phenolic hydroxyl-group content by 0·35. The lignin of mature *E. regnans* does not differ significantly from this.

4. An approximately equal number of syringyl and guaiacyl units are present in the lignin of *E. regnans*.

5. Potentiometric determination indicates that one in three of the C_9 units is phenolic and ionized in dimethylformamide, whereas spectrophotometric determination shows one in five to be ionized in methanolic 0·1 N-potassium hydroxide.

6. The spectrophotometric data suggest that of the phenolic groups ionized in the 0·1 N-potassium hydroxide 60% are syringyl groups.

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REFERENCES

Adler, E. (1958). *Proc. 4th int. Congr. Biochem., Vienna*, vol. 2, p. 137.
Adler, E. & Gierer, J. (1955). *Acta chem. scand.* **9**, 84.
Arlt, H. G., Sarkanen, K. & Schuerch, C. (1956). *J. Amer. chem. Soc.* **78**, 1904.
Aulin-Erdtman, G. (1953*a*). *Svensk Papp-Tidn.* **56**, 91.
Aulin-Erdtman, G. (1953*b*). *Svensk Papp-Tidn.* **56**, 287.
Aulin-Erdtman, G. (1954). *Svensk Papp-Tidn.* **57**, 745.
Aulin-Erdtman, G. (1957). *Svensk Papp-Tidn.* **60**, 671.
Björkman, A. & Person, B. (1957). *Svensk Papp-Tidn.* **60**, 158.
Bland, D. E. (1958*a*). *Holzforschung*, **12**, 19.
Bland, D. E. (1958*b*). *Holzforschung*, **12**, 36.
Bland, D. E., Billek, G., Gruber, K. & Kratzl, K. (1959). *Holzforschung*, **13**, 6.
Bland, D. E. & Gatley, F. M. (1954). *Nature, Lond.*, **173**, 32.
Bland, D. E., Ho, G. & Cohen, W. E. (1950). *Aust. J. sci. Res.* **3A**, 642.
Bland, D. E. & Stamp, C. (1955). *Aust. J. appl. Sci.* **6**, 353.
Brauns, F. E. (1952). *The Chemistry of Lignin*, p. 14. New York: Academic Press Inc.
Butler, J. P. & Czepiel, T. P. (1956). *Analyt. Chem.* **28**, 1468.
Enkvist, T., Alm, B. & Holm, B. (1956). *Paperija Puu*, **38**, 1.
Erdtman, H., Lindgren, B. O. & Petterson, T. (1950). *Acta chem. scand.* **4**, 228.
Freudenberg, K. & Dall, K. (1955). *Naturwissenschaften*, **22**, 606.
Freudenberg, K. & Schlüter, H. (1955). *Chem. Ber.* **88**, 617.
Gierer, J. (1954). *Acta chem. scand.* **8**, 1319.
Higgins, H. G. (1957). *Aust. J. Chem.* **10**, 496.
Hillis, W. E. (1954). *J. Soc. Leath. Tr. Chem.* **38**, 177.
Merewether, J. W. T. (1954). *T.A.P.P.I.*, **37**, 483.
Sarkanen, K. & Schuerch, C. (1957). *J. Amer. chem. Soc.* **79**, 4203.

Biochem. J. (1960) **75**, 201

The Spontaneous and Induced Recovery of Fly-Brain Cholinesterase after Inhibition by Organophosphates

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The widely held belief is that organophosphates exert their insecticidal action through the inhibition of cholinesterase (Spencer & O'Brien, 1957; Fukuto, 1957). Mengle & Casida (1958), with 17 organophosphates in amounts corresponding to the LD_{50}, showed that cholinesterase *in vivo* was usually largely inhibited within a few hours, but always recovered in the survivors to 90% or more of normal within 1 day. These recoveries are greater than those found in the mammal *in vivo* (Davison, 1955). There appeared to be no connexion between the nature of the alkoxy substituents and

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rate of recovery, as there is in mammals (Davison, 1955).

The validity of the data of Mengle & Casida (1958) was brought into doubt by the findings of Van Asperen (1958*a*), who made the point that workers measuring cholinesterase inhibition often obtained erroneous results. This was based upon the assumption that excess of inhibitor present in the tissue, but not bound to the enzyme, came in contact, especially during homogenization, with uninhibited enzyme. To avoid this effect Van Asperen homogenized whole flies directly in acetylcholine solution after 'knock down' (i.e. severe paralysis) with dimethyl-2:2-dichlorovinyl phosphate vapours. In this way there was no further inhibition of the enzyme because uninhibited enzyme was 'protected' by the acetylcholine from inhibition.

In the present study similar protection techniques were used and the results were compared with those obtained by the standard method of Mengle & Casida (1958). The comparison shows no difference between results obtained in the two methods and indicates that the rapid inhibition *in vivo* and recovery of cholinesterase activity is a real phenomenon.

In the mammal, spontaneous recovery *in vitro* of inhibited cholinesterase is well known (Hobbiger, 1951; Aldridge, 1953; Aldridge & Davison, 1953). Yet in the housefly Van Asperen (1958*b*) reported no spontaneous recovery *in vitro* after inhibition with dimethyl-2:2-dichlorovinyl phosphate. In the present work this compound and four additional organophosphate compounds were used to inhibit fly-head cholinesterase *in vitro* and with all five there was no spontaneous recovery. This failure to recover *in vitro* could be due to rapid 'ageing' of the inhibited cholinesterase to an irreversible form, as was reported to occur in mammals (Hobbiger, 1956; Davies & Green, 1956). This possibility has been examined in the present work by the use of potent cholinesterase reactivators *in vitro* and *in vivo*.

The paradoxical difference between effects *in vivo* and *in vitro* is examined below, and a possible explanation is proposed.

MATERIALS AND METHODS

Adult female houseflies, *Musca domestica* L. (CSMA-1948 strain), were used the day after emergence.

The organophosphates used were: malathion, *S*-1:2-di-(ethoxycarbonyl)ethyl *OO*-dimethyl phosphorothiolothionate; malaoxon, *S*-1:2-di(ethoxycarbonyl)ethyl *OO*-dimethyl phosphorothiolate; parathion, diethyl *p*-nitrophenyl phosphorothionate; methyl parathion, dimethyl *p*-nitrophenyl phosphorothionate; paraoxon, diethyl *p*-nitrophenyl phosphate; tetraethyl pyrophosphate (TEPP); dimethyl 2:2-dichlorovinyl phosphate (DDVP); dimethoate (American Cyanamid Co.), *OO*-dimethyl *S*-(*N*-methyl carbamoylmethyl) phosphorodithioate; di*iso*propyl phosphorofluoridate (DFP).

Studies in vivo

Flies were treated on the ventral tip of the abdomen with the predetermined 1280 min. LD_{50} of the organophosphate compound in acetone. These were as follows (μg./g.): DFP, 6·0; TEPP, 5·0; paraoxon, 0·75; malathion, 15; parathion, 1·0; dimethoate, 0·45; DDVP, 1·5; methyl parathion, 1·0. At various periods up to 1280 min. after treatment the surviving flies were decapitated and the cholinesterase activity was determined by a standard manometric technique (Mengle & Casida, 1958) and by the following protective technique: fly heads were homogenized at 0° at a concentration of 1·5 heads/ml. in 0·01 M-acetylcholine bromide in buffer (0·025 M-$NaHCO_3$, 0·15 M-NaCl, 0·04 M-$MgCl_2$). A portion (2 ml.) of this preparation was added at zero time to Warburg flasks, which were then transferred to the bath at 38°, gassed with $CO_2 + N_2$ (5:95), equilibrated and readings were begun 24 min. from zero time. In a few cases 4 heads/ml. were used, and it was then necessary to tip in an additional amount of acetylcholine (1·6 mg. in 0·4 ml. in the side arm) just before the zero readings.

Studies in vitro

Solutions of organophosphates in dilute (1% or less) ethanol were added to homogenates of fly heads in buffer (as described above, without acetylcholine) at concentrations predetermined to inhibit 95% or more of the cholinesterase activity after incubation for 1 hr. at 38°. The excess of inhibitor in the homogenate was then removed by adding a preparation of rabbit-plasma A-esterase (Aldridge, 1953) and incubating additionally for 1 hr. at 38°. (This A-esterase preparation was made by adding 1 ml. of 0·1 mM-DFP to 10 ml. of heparinized rabbit plasma, incubating for 1 hr. at 38° and storing at 5° until used; it contained no cholinesterase activity even after 24 hr. storage or after addition of oximes.) Experiments showed that this treatment totally eliminated excess of inhibitor.

At 1, 4, 12 and 24 hr. after the end of the inhibition period groups of samples were removed: one pair of samples was assayed for cholinesterase to determine spontaneous recovery. Each sample was assayed in triplicate. To other pairs were added various oximes, and cholinesterase was determined 1 hr. later; this gave the oxime-reactivatable cholinesterase. In these experiments magnesium was omitted from the buffer since it inhibits A-esterase (Aldridge, 1953). Also investigated was the recovery *in vitro*, both spontaneous and oxime-induced, of fly-head cholinesterase inhibited *in vivo*. In this study flies were treated as described above, but at the predetermined time of maximum cholinesterase inhibition they were decapitated, the heads were homogenized and the resulting brei was studied for spontaneous and oxime-induced recovery of enzyme activity.

RESULTS AND DISCUSSION

Cholinesterase inhibition and recovery in vivo

The possibility that the previously reported inhibitions *in vivo* (Mengle & Casida, 1958) were high owing to artifacts was examined by comparing the protective with the standard procedure

at the time of maximum cholinesterase inhibition. As Table 1 shows, the protective technique did not give higher estimates of the cholinesterase for the three phosphorothionates studied.

Another approach was used: the possibility of an excess of inhibitor in the heads from treated flies was examined by homogenizing these heads with those from untreated flies. Preliminary experiments showed a linear relationship between enzyme concentration and activity under these conditions. As Table 2 shows, excess of inhibitor was found only at $10 \times LD_{50}$ of DDVP and parathion. At the LD_{50} for DDVP and TEPP and at $10 \times LD_{50}$ for TEPP the activities were higher than expected, suggesting that the heads of the untreated flies contained some form of reactivating factor. A similar conclusion may be drawn from the data of Rasmuson & Holmstedt (1958) for parathion and tabun (*O*-ethyl *NN*-dimethylphosphorocyanamidate) with the housefly.

With this technique, excess of inhibitor was shown by Van Asperen (1957) in houseflies with DDVP: the concentration of inhibitor fell to one-half after 22 hr. Colhoun (1959) found excess of TEPP for 7 hr. after treatment of American cockroaches. Rasmuson & Holmstedt (1958) found excess of paraoxon in houseflies at 'knock down'.

We conclude that at the LD_{50} phosphorothionates have not shown the homogenization artifact. Phosphates may show artifacts when used at high concentrations. Probably our technique of treatment on the tip of the abdomen and assay of cholinesterase only in the head minimizes the artifact: Van Asperen (1957) found that the head contained far less inhibitor than the body after vapour treatment by DDVP.

The studies on the time course of inhibition *in vivo* (Mengle & Casida, 1958) were extended to three more compounds: DFP, TEPP and paraoxon at the LD_{50} (Fig. 1). All showed similar effects. It was particularly surprising that the cholinesterase of DFP-treated flies recovered so readily. A check with the protective method showed that the severe inhibition and recovery was genuine. When DFP at the LD_{75} was used, recovery in the survivors to only 15–20 % of normal was noted in 1280 min. Recovery could have been more extensive later on.

Table 1. *Cholinesterase activity in fly heads at various times after treatment* in vivo *with malathion, parathion or dimethoate*

Each figure is the mean of triplicate Warburg assays. The LD_{50} dose was given to each fly. Cholinesterase was determined as described in the Materials and Methods section.

Insecticide	Time after treatment (min.)	Heads/ml.	Percentage of pretreatment cholinesterase activity: Protective method	Percentage of pretreatment cholinesterase activity: Standard method
Malathion	40	1·5	23, 25	26, 32
	40	4·0	16, 21	27, 28
Parathion	160	1·5	8, 15	11, 17
	160	4·0	25, 27	23, 29
Dimethoate	160	4·0	21, 29	22, 24

Table 2. *Examination for excess of inhibitor in heads of treated flies*

Flies were decapitated 1 hr. after treatment with the topical LD_{50} dose or 10 times the LD_{50} dose. Equal numbers of treated and untreated heads were mixed, homogenized by the standard (non-protective) technique at 1·5 heads/ml., and the homogenate was kept for 1 hr. at 38°. Cholinesterase activity was assayed as described in the Materials and Methods section.

Compound	Percentage of normal cholinesterase activity: At LD_{50}: Treated	At LD_{50}: Treated + untreated (observed)	At LD_{50}: Treated + untreated (predicted)*	At $10 \times LD_{50}$: Treated	At $10 \times LD_{50}$: Treated + untreated (observed)	At $10 \times LD_{50}$: Treated + untreated (predicted)*
DDVP	6	63	53	5	7	53
TEPP	5	91	53	0	65	50
Parathion	5	55	53	6	33	53
Malathion	26	61	63	15	59	58

* Prediction based on the assumption of no excess of inhibitor in heads of treated flies. If prediction exceeds observation, excess of inhibitor is present. If observation exceeds prediction, reactivation is suggested.

Cholinesterase recovery in vitro *after inhibition* in vitro

The observation by Van Asperen (1958*b*) that housefly cholinesterase did not recover spontaneously *in vitro* after treatment with DDVP was confirmed and extended to four other inhibitors. Human-plasma cholinesterase was shown to reverse readily, as is well known (Table 3). Our data on plasma show the spontaneous recovery to depend on the alkoxy substituent in the descending order ethyl, methyl, *iso*propyl. Aldridge (1953) showed that the order was methyl, ethyl for rabbit whole-blood cholinesterase.

The phenomenon of 'ageing' has been found with both mammalian cholinesterases: true and pseudo-phosphorylated cholinesterase slowly changes into a form which reactivates neither spontaneously nor under the influence of oximes (Hobbiger, 1956; Davies & Green, 1956). To study the possibility that rapid ageing accounted for the failure of spontaneous recovery in fly cholinesterase, the effect of oximes was studied with fly-head cholinesterase inhibited *in vitro* by excess of organophosphate (0·1 mM) subsequently removed by A-esterase.

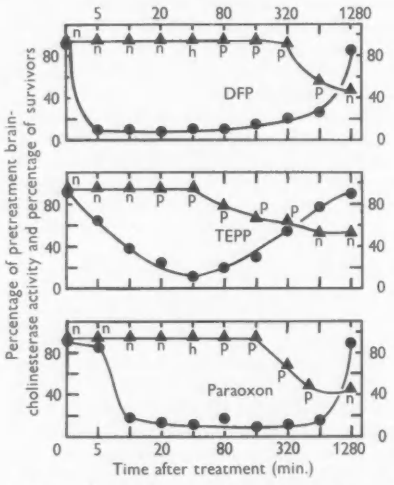

Fig. 1. Comparison of time mortality and symptomatology curves with fly-brain cholinesterase activity of surviving flies, with three organophosphates. Symptoms observed at predetermined times after treatment were: n, normal; h, hyperactivity; p, paralysis. ●, Percentage of pretreatment brain-cholinesterase activity; ▲, percentage of survivors.

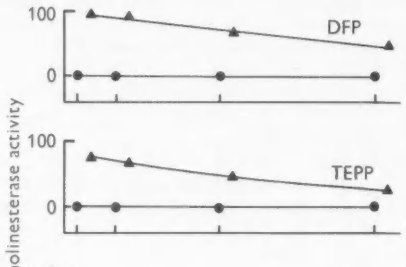

Fig. 2. Recovery *in vitro* of fly-brain cholinesterase inhibited *in vitro*. The various organophosphate inhibitors were added at 0·1 mM and excess was later removed with an A-esterase preparation. ▲, After 1 hr. with 2 mM-pyridine 2-aldoxime methiodide; ●, no oxime added.

Table 3. *Spontaneous recovery of cholinesterase* in vitro *in 24 hr.*

Values expressed are percentages of normal (uninhibited) cholinesterase activity observed after 1 hr. incubation with 0·1 mM inhibitor at 38° with subsequent removal of excess of inhibitor and holding at 38° for 24 hr. Excess of inhibitor was removed by adding the A-esterase preparation at the rate of 1 ml. of A-esterase to 9 ml. of the inhibited tissue homogenate. After the holding period cholinesterase activity was determined by the standard method. These values were compared with the cholinesterase activity of uninhibited homogenates treated only with the A-esterase preparation and held for 24 hr. before assay. Before the holding period the cholinesterase activity of the inhibited homogenate was in all cases less than 5% of that found in the uninhibited homogenate.

Origin of enzyme	Inhibitor: DFP	TEPP	Paraoxon	Malaoxon	DDVP
	Percentage of uninhibited cholinesterase activity				
Human plasma	2–7	41–46	54–58	19–21	40–46
Fly head	0	1	0	0	0

Fig. 2 shows results with pyridine 2-aldoxime methiodide: similar but far less effective results were found with pyridine 2-aldoxime dodecaiodide. For instance, a portion of one sample of cholinesterase which had been freshly inhibited by DFP could be restored to 94% of normal by pyridine 2-aldoxime methiodide, but another portion of this sample could be restored only to 6% of normal by pyridine 2-aldoxime dodecaiodide. With di*iso*nitrosoacetone monodibutylamine salt, no recovery was obtained. The behaviour with pyridine 2-aldoxime methiodide was as with the mammalian enzyme: the enzyme could be largely reactivated at first, but slowly became less capable of reactivation on standing. 'Ageing' with respect to oximes therefore occurs. The 'ageing' rate depends on alkoxy substituents, being in the descending order methyl, ethyl, *iso*propyl. The corresponding order in erythrocyte cholinesterase is methyl (= *iso*propyl), ethyl (Davies & Green, 1956). 'Ageing' of housefly cholinesterase *in vitro* is not an extremely rapid process, and cannot account for the failure of the inhibited enzyme to recover spontaneously.

Comparison of recovery in vivo *and* in vitro

We have an interesting paradox: after organophosphate poisoning, cholinesterase *in vivo* recovers more rapidly than does that of the mammal (Davison, 1955). Yet after inhibition *in vitro* housefly cholinesterase shows no spontaneous recovery, in contrast with the mammal.

Two hypotheses are possible: (*a*) The nature of the inhibited cholinesterase *in vivo* may be different from that inhibited *in vitro*, being more reversible. This was tested by homogenizing treated flies at the time of maximum cholinesterase depression.

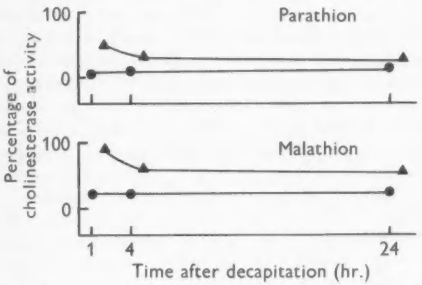

Fig. 3. Recovery *in vitro* of fly-brain cholinesterase inhibited *in vivo*. The organophosphates were applied at the LD_{50} dose and the flies were decapitated at the predetermined time of maximum cholinesterase inhibition. Time from treatment to decapitation for parathion was 160 min.; for malathion the time was 40 min. ▲, After 1 hr. with 2 mM-pyridine 2-aldoxime methiodide; ●, no oxime added.

This inhibited cholinesterase behaved precisely as did enzyme inhibited *in vitro*: it did not recover at all spontaneously; it was readily reactivated by oximes at first; and it 'aged' to a less reactivatable form (Fig. 3). This hypothesis is therefore not tenable.

(*b*) There is in intact flies a reactivating factor, which is partially destroyed by homogenization. Such a factor has been shown in mammalian sera by Neubert, Schaefer & Kewitz (1958). There are two pieces of evidence for the occurrence of this factor in flies. First, as shown in Table 2, homogenates of mixed heads from treated and untreated flies may give cholinesterase activity in excess of that predicted on the assumption that the inhibited cholinesterase is not affected by the process. Secondly, fly-head cholinesterase was treated *in vitro* with various inhibitors to obtain total inhibition. Then after various times fresh preparations of untreated fly (whole insects or heads only) were added. As Table 4 shows, these preparations were quite effective in reactivating the inhibited cholinesterase. As with oximes, the capacity of the inhibited enzyme to be reactivated decreased with time; by 12 hr. little reactivation was possible. The reactivating capacity of the untreated fly preparations was rapidly lost on standing; after 30 min. in the refrigerator it was completely lost.

Experiments showed that the cholinesterase of flies killed by organophosphate poisoning was almost totally inhibited, and did not recover upon standing at room temperature, e.g. with the LD_{50} of parathion it was 6% of normal, and 15 hr. later was 2%; with malathion the figures were 5 and 0%, with TEPP 4 and 0%. In earlier work (Mengle & Casida, 1958) with Dipterex (dimethyl 2:2:2-trichloro-1-hydroxyethylphosphonate) and Thimet (*OO*-diethyl *S*-ethylthiomethyl phosphorodithioate) cholinesterase in dead flies was not so substantially inhibited at sampling and showed a little spontaneous reactivation, e.g. from 15 to 27% in 15 hr. with Thimet. For malathion O'Brien (1956) found that 24 hr. after the LD_{50} of malathion the total population (dead and alive) had 50% of normal cholinesterase; taken with Mengle & Casida's observation that under similar conditions the live flies had 100% of their normal cholinesterase, this suggests that at 24 hr. the cholinesterase of the dead flies was totally inhibited; since inhibition was maximal at 20 min., the cholinesterase of the dead flies cannot have recovered at all.

We can conclude that there exists in living flies a factor which can reactivate inhibited cholinesterase. This factor is very labile after homogenization and is presumably of reduced effectiveness in flies killed by organophosphates, since their cholinesterase fails to recover.

Table 4. *Determination of reactivation of inhibited fly-brain cholinesterase by homogenates of untreated heads and whole flies*

Data are expressed as μl. of CO_2 evolved/20 min. in excess of predicted value, which is the mean of that for inhibited homogenate and that for untreated homogenate. The predicted value for each concentration of fly homogenate is shown in the bottom line. Treated homogenate contained 1·5 fly heads/ml. of solution. Inhibition was with 0·1 mM inhibitor for 1 hr. at 38°. After inhibition the excess of inhibitor was removed by adding A-esterase preparation and incubating at 38°. At periods of 1, 12 and 24 hr. after adding A-esterase the treated homogenates were added at the rate of 1 ml. to 1 ml. of homogenate of untreated fly heads or whole flies, the concentration of which is given in the Table. The inhibited and fresh tissue homogenates were mixed at 38° for 1 hr., after which cholinesterase determinations were made upon 1·8 ml. portions of the mixture.

Treatment *in vitro* (hr. after inhibition)	Untreated homogenate			
	1·5 heads/ml.	1·5 flies/ml.	0·15 head/ml.	0·15 fly/ml.
DFP				
1	46	51	31	27
12	5	23	11	10
24	0	5	0	0
TEPP				
1	34	26	24	28
12	0	5	4	7
24	0	1	0	1
Paroxon				
1	25	17	16	12
12	1	5	5	8
24	0	4	0	0
Malaoxon				
1	46	54	11	9
12	13	8	5	0
24	0	1	0	0
Predicted values ...	72	81	11	13

If endogenous reactivating factors assist in the reversal of cholinesterase inhibition *in vivo*, administered oximes might possibly hasten the process and thus reduce the toxicity of organophosphates. Parathion, methyl parathion, paraoxon, malathion, DFP and TEPP were applied to the abdomen at the LD_{50}, $2 \times LD_{50}$ and $8 \times LD_{50}$ doses. The oximes injected simultaneously were pyridine 2-aldoxime methiodide, pyridine 2-aldoxime dodecaiodide or di*iso*nitrosoacetone at 50 μg./g. and 1000 μg./g. No effect on the mortality at 24 hr. was found except for DFP, whose LD_{50} was increased 1·5-fold by both levels of pyridine 2-aldoxime methiodide. Symptoms were delayed for about 1 hr. with parathion, malathion and DFP at both concentrations of pyridine 2-aldoxime methiodide. No substantial changes were induced by pyridine 2-aldoxime methiodide (500 μg./g.) in the pattern of cholinesterase inhibition *in vivo* after poisoning with malathion and parathion. This was equally true if the oxime was injected 2 hr. before the organophosphate, simultaneously with it, or near the pre-established period of maximal inhibition.

Winteringham (1957) and Winteringham, Harrison, McKay and Weatherley (1957), using DFP and pyridine 2-aldoxime methiodide (PAM), have reported alleviation of the symptoms of paralysis and respiratory stimulation, but no effect on LD_{50}. Our different finding for LD_{50} is not seriously in conflict with theirs, since a 1·5-fold increase in LD_{50} is of little importance.

Winteringham (1957) concluded: 'It seems, therefore, that PAM successfully reversed the anticholinesterase action in the insect as indicated by its recovery from paralysis but failed to protect the insect from the slower lethal effect.' He therefore assumes that paralysis is due to cholinesterase inhibition, but that death is not. Our data suggest the contrary: since the oximes have no marked effect *in vivo* either upon cholinesterase or mortality, cholinesterase inhibition must still be considered a reasonable cause of death. Pyridine 2-aldoxime alleviates paralysis, which may therefore be caused by inhibition of some system other than cholinesterase. At 'knock down' Van Asperen (1957) showed that aliesterase was severely inhibited; D. Stegwee (personal communication) finds that aliesterase is located principally outside the nerve cord of the housefly and would therefore be accessible to ionized compounds such as pyridine 2-aldoxime. It is therefore possible that paralysis and respiratory stimulation are caused by inhibition of aliesterase (or related enzymes) but that this inhibition is not important in causing death.

The above-described effects of oximes *in vivo* are smaller than those found for mammals (Kewitz, Wilson & Nachmansohn, 1956; Hobbiger, 1957). The discrepancy is probably attributable to ionization in pyridine 2-aldoxime methiodide and di*iso*nitrosoacetone; ionized compounds fail to penetrate to the nervous system of insects (Twarog & Roeder, 1957; Winton, Metcalf & Fukuto, 1958; O'Brien & Fisher, 1958). Di*iso*nitrosoacetone was found to have pK_a 10·5, and therefore is 99·9% ionized at pH 7·0. Although pyridine 2-aldoxime dodecaiodide is much more soluble in lipids than is the methiodide (Wilson, 1958), this property did not facilitate its action in the fly, in fact the use of dodecaiodide did not show the small alleviation found with the methiodide.

SUMMARY

1. With the six organophosphates tested, the brain cholinesterase of poisoned houseflies is rapidly inhibited. In the survivors, this cholinesterase recovers almost to normal within 1 day; in dead flies, it remains almost fully inhibited. These results are not due to homogenization artifacts.

2. The cholinesterase of the housefly differs from both true and pseudo-cholinesterase of mammals in that spontaneous recovery does not occur after inhibition by organophosphates *in vitro*. It can be reactivated by oximes. Its capacity to be reactivated slowly disappears at a rate depending upon the alkoxy substituents of the organophosphates: methyl > ethyl > *iso*propyl. This order differs from that found with mammalian cholinesterase.

3. Housefly-brain cholinesterase inhibited *in vivo* behaves *in vitro* precisely as that inhibited *in vitro*.

4. The discrepancy between ready recovery of cholinesterase *in vivo* and failure to recover *in vitro* is attributed to a labile reactivating factor present in living flies and fresh homogenates of untreated flies.

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REFERENCES

Aldridge, W. N. (1953). *Biochem. J.* **54**, 442.
Aldridge, W. N. & Davison, A. N. (1953). *Biochem. J.* **55**, 763.
Colhoun, E. H. (1959). *Canad. J. Biochem. Physiol.* **37**, 259.
Davies, D. R. & Green, A L. (1956). *Biochem. J.* **63**, 529.
Davison, A. N. (1955). *Biochem. J.* **60**, 339.
Fukuto, T. R. (1957). *Advanc. Pest Control Res.* **1**, 147.
Hobbiger, F. (1951). *Brit. J. Pharmacol.* **6**, 21.
Hobbiger, F. (1956). *Brit. J. Pharmacol.* **11**, 295.
Hobbiger, F. (1957). *Brit. J. Pharmacol.* **12**, 438.
Kewitz, H., Wilson, I. B. & Nachmansohn, D. (1956). *Arch. Biochem. Biophys.* **64**, 456.
Mengle, D. C. & Casida, J. E. (1958). *J. econ. Ent.* **51**, 750.
Neubert, D., Schaefer, J. & Kewitz, H. (1958). *Naturwissenschaften*, **45**, 290.
O'Brien, R. D. (1956). *J. econ. Ent.* **49**, 484.
O'Brien, R. D. & Fisher, R. W. (1958). *J. econ. Ent.* **51**, 169.
Rasmuson, B. & Holmstedt. B. (1958). *LantbrHögsk. Ann.* **24**, 89.
Spencer, E. Y. & O'Brien, R. D. (1957). *Annu. Rev. Ent.* **2**, 261.
Twarog, B. M. & Roeder, K. D. (1957). *Ann. ent. Soc. Amer.* **50**, 231.
Van Asperen, K. (1957). *4th int. Congr. Pl. Prot., Hamburg.*
Van Asperen, K. (1958*a*). *Nature, Lond.*, **181**, 355.
Van Asperen, K. (1958*b*). *Biochim. biophys. Acta*, **28**, 603.
Wilson, I. B. (1958). *Biochim. biophys. Acta*, **27**, 196.
Winteringham, F. P. W. (1957). *Chem. & Ind.* p. 1195.
Winteringham, F. P. W., Harrison, A., McKay, M. A. & Weatherley, A. (1957). *Biochem. J.* **66**, 49P.
Winton, M. Y., Metcalf, R. L. & Fukuto, T. R. (1958). *Ann. ent. Soc. Amer.* **51**, 436.